

Institut für Veterinärphysiologie
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. med. vet. M. Gassmann
Arbeitsgruppe Prof. Dr. med. vet. Thomas A. Lutz

Arbeit unter Leitung von PD Dr. T. Riediger

Involvement of nitric oxide in disease-related anorexia

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Caroline Cordani

Tierärztin
von Wangen, SZ

genehmigt auf Antrag von

PD Dr. Thomas Riediger, Referent

PD Dr. Herbert Schmid, Korreferent

Zürich 2010

Contents

2 Introduction.....	- 4 -
2.1 Nitric oxide	- 5 -
2.2 NO formation in the hypothalamic arcuate nucleus (ARC).....	- 6 -
2.3 The area postrema / nucleus tractus solitarii region.....	- 7 -
2.4 The JAK-STAT pathway	- 8 -
2.5 NO-based drug therapy	- 9 -
2.6 Aim and Hypothesis.....	- 10 -
3 Material and Methods	- 12 -
3.1 Animals and housing.....	- 12 -
3.2 Transmitter surgery	- 12 -
3.3 Metabolic cages (PhysioScan System)	- 13 -
3.4 Diets	- 14 -
3.5 Food and water consumption	- 14 -
3.6 Body temperature and physical activity measurements.....	- 15 -
3.7 Perfusion and immunohistochemistry.....	- 15 -
3.8 Experimental paradigms	- 16 -
3.8.1 Effect of 1400W on LPS-induced anorexia and associated disease symptoms	- 16 -
3.8.2 Time course of LPS-induced pSTAT3 formation.....	- 17 -
3.8.3 Temporal association between pSTAT3 formation and LPS-induced anorexia.....	- 17 -
3.9 Statistical analyses	- 18 -
4 Results	- 20 -
4.1 Effect of 1400W on LPS-induced anorexia and associated disease symptoms..	- 20 -
4.2 Time course of LPS-induced pSTAT3 formation.....	- 28 -
4.3 Temporal association between pSTAT formation and LPS-induced anorexia...	- 31 -
5 Discussion	- 36 -
5.1 Effect of 1400W treatment on LPS-induced anorexia and associated disease symptoms	- 36 -
5.2 LPS-induced pSTAT3 formation in the ARC.....	- 39 -
5.3 Association between pSTAT formation and LPS-induced anorexia	- 41 -
5.4 Interaction between the pSTAT/iNOS pathway and COX-2 signaling.....	- 42 -
5.5 Proposed model.....	- 43 -
6 References.....	- 45 -

Summary

The nitric oxide (NO) producing enzyme inducible NO synthase isoform (iNOS) is strongly upregulated under inflammatory conditions in the arcuate nucleus, a hypothalamic key structure for the control of food intake and energy balance. This project aims to prove the concept that a pharmacological blockade of iNOS attenuates anorexia and associated disease symptoms induced by the proinflammatory endotoxin lipopolysaccharide (LPS). We also analyzed the association between LPS-induced anorexia and pSTAT3-formation in the arcuate nucleus and in hindbrain feeding centers (AP/NTS region). pSTAT3 is a cytokine dependent transcription factor, which is suggested to control iNOS expression.

Findings and conclusions:

- ❖ NO contributes to LPS-induced anorexia because anorexia is attenuated by the specific iNOS-inhibitor 1400W. In addition, 1400W treatment also ameliorates other LPS-induced symptoms (e.g. adipisia, fever, inactivity).
- ❖ LPS treatment induces a time-dependent pSTAT3 formation in the ARC and the AP/NTS region of rats; this parallels the time course of LPS anorexia.
- ❖ LPS does not induce a pSTAT3 response in LPS tolerant rats that do not show an anorectic LPS response either.

These findings provide evidence that NO is involved in disease-related anorexia and that pharmacological iNOS blockade may be a therapeutic approach to treat sickness anorexia/cachexia. LPS anorexia is associated with enhanced pSTAT3 signaling. Which might be involved in the underlying transcriptional mechanisms.

2 Introduction

Disease-related anorexia is a detrimental factor caused by an inflammatory state associated with several acute and chronic diseases such as bacterial and pulmonary infection, cancer, AIDS, chronic obstructive pulmonary disease (COPD), chronic inflammatory bowel disease and rheumatoid arthritis (Plata-Salaman 2000). Anorexia in disease is part of the generalized host defense termed “acute-phase response” (APR) that at the behavioral level includes pronounced hypophagia, fever, reduction in locomotion, adipsia, altered sleep patterns, reduction in sexual interest and in self grooming. These alterations do not represent an exhaustion of the host, instead, they are centrally organized and are thought to facilitate recovery (Kinoshita et al. 2009).

Anorexia during disease can be beneficial or deleterious depending on its timing and duration. Transient anorexia during acute disease may be beneficial to an organism since a restriction in nutrient intake will delay bacterial growth. Sustained anorexia during chronic disease, however, is harmful to an organism and may be linked to cachexia and a negative impact on recovery, which may ultimately result in increased mortality (Plata-Salaman 1996). E.g. up to 50% of cancer patients are affected by a progressive atrophy of adipose tissue and skeletal muscle, called cachexia, resulting in weight loss, reduced quality of life and a shortened survival time. Depending on the tumor type, weight loss occurs in 30–80% of cancer patients and is severe (with loss of >10% of the initial body weight) in 15%. Patients with pancreatic or gastric cancer show the highest frequency of weight loss (Tisdale 2009).

Injection of microbial products such as lipopolysaccharides (LPS) is often used to simulate acute inflammation and to study disease-related anorexia. LPS is a major constituent of the cell wall of gram-negative bacteria and represents a target for the immune system. Exposure to LPS triggers the acute-phase response and causes anorexia mainly through the release of pro-inflammatory cytokines (Langhans 2007). These cytokines – mainly interleukin 1 and 6, tumor necrosis factor alpha and interferon – play a pivotal role in the development of disease-related anorexia. Each of these cytokines reduces food intake after peripheral or central administration (Langhans and Hrupka 1999) and cytokine antagonism by receptor blockade or inhibition of cytokine production has been shown to reduce sickness-related anorexia (Porter et al. 2000).

Of note, the anorectic response to LPS is not a direct consequence of the fever response. This is supported by the demonstrations that genetic disruption of interleukin-6 (IL-6) prevents the LPS-induced fever response (Chai et al. 1996) while IL-6 deficient mice

treated with LPS still show anorexia (Fattori et al. 1994).

Once released from peripheral immune cells (such as blood monocytes, tissue macrophages and others), cytokines act on the brain via two communication pathways: First, via a neuronal pathway i.e. afferent branches of the vagus nerve and second via a humoral pathway that involves the diffusion of cytokines from the peripheral circulation into the brain parenchyma of circumventricular organs lacking a blood-brain barrier. Furthermore, cytokines may act on the vascular endothelium and induce the formation of secondary messengers such as prostaglandins (via induction of the enzyme cyclooxygenase (COX)) or nitric oxide (Reyes and Sawchenko 2002; Dantzer 2006). For example, prostaglandin E, which is released from the organum vasculosum of the lamina terminalis (OVLT) in response to endogenous pyrogens, acts on thermoregulatory neurons of the preoptic anterior hypothalamic area to bring about the fever response (Stitt and Shimada 1989).

2.1 Nitric oxide

NO is a gaseous signaling molecule that is involved in various physiological and pathophysiological functions in the body, including the control of blood circulation and blood pressure, platelet function, host defense, neuronal activity, neurotransmission and others (Korhonen et al. 2005).

NO is synthesized from L-arginine by the enzyme NO synthase (NOS), resulting in the formation of L-citrullin and NO. In mammalian cells three different NOS isoforms have been identified. The neuronal NOS (nNOS) is expressed in neurons in the central and peripheral nervous system and is involved in cell-cell communication. NO produced by endothelial NOS (eNOS) is released from endothelial cells and leads to vasodilatation. Both nNOS and eNOS are constitutively expressed and are inactive in resting cells. An increase in free intracellular calcium activates these enzymes (Korhonen et al. 2005). Another NOS isoform is the inducible NOS (iNOS), which is synthesized and released in response to inflammatory stimuli such as LPS or proinflammatory cytokines. Contrary to the other NOS isoforms, iNOS activity is independent of the intracellular calcium concentration (Sparrow 1994; Korhonen et al. 2005). In the brain iNOS seems to be expressed in glial cells, endothelial cells, neurons and microglia cells in response to LPS or pro-inflammatory cytokines (Moncada et al. 1991; Minghetti et al. 1997).

NO is a highly lipophilic neuromodulator, which can freely diffuse across cell membranes. The biological half life of NO seems to be only a few seconds (Stamler et al. 1992). The best investigated cellular function of NO is to activate the cytoplasmatic

enzyme soluble guanylate cyclase (sGC) in neighbouring cells, which catalyzes the formation of the intracellular second messenger cyclic guanosine monophosphate (cGMP) (Ignarro 1991).

One of the best documented functions of NO in the context of inflammatory processes is its critical role in the induction of fever in different species (Parrott et al. 1998; Roth et al. 1998). A pharmacological or genetic disruption of NO formation attenuates LPS-mediated induction of fever (Soszynski 2001; Kozak and Kozak 2003).

Previous studies suggest that the induction of iNOS during inflammation and the subsequent release of NO in the hypothalamic arcuate nucleus might also be an important factor in the development of disease-related anorexia.

2.2 NO formation in the hypothalamic arcuate nucleus (ARC)

The arcuate nucleus is one of the most important hypothalamic structures involved in the control of food intake and energy homeostasis. It is located at the basis of the third ventricle and neighbours the median eminence, and it contains two functionally distinct groups of neurons releasing different types of neuropeptides: orexigenic neurons expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP), which both stimulate food intake and which in the rat are mainly localized in the medial part of the ARC; anorexigenic neurons expressing pro-opiomelanocortin (POMC) producing the anorectic neuropeptide α -MSH and cocaine-amphetamine-related transcript (CART) and which in the rat are mainly localized in the lateral part of the ARC. The axons of both types of neurons project to “second-order” neurons located in the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA) (Barsh and Schwartz 2002; Valassi et al. 2008).

The best-investigated role of the ARC is its receptive function for leptin, which is secreted from adipocytes in relation to the body’s fat stores. Leptin has a dual action in the ARC; it activates POMC neurons and upregulates the expression and release of α -MSH (Cowley et al. 2001), and it inhibits NPY neurons and NPY expression (Spanswick et al. 1997; Cowley et al. 2001).

A functional antagonist of leptin in the ARC seems to be the orexigenic hormone ghrelin, which is mainly released from the stomach (Kojima et al. 1999). Ghrelin directly activates leptin-inhibited ARC neurons (Traebert et al. 2002) and it stimulates the synthesis of NPY and AgRP in the ARC; when injected centrally or peripherally, it enhances food intake and adiposity in rodents (Tschop et al. 2000; Horvath et al. 2001; Wren et al. 2001).

In addition to the receptive function of the ARC for hormones involved in the control of energy balance, there is evidence that NO formation in the ARC may contribute to disease-related anorexia. While no measurable iNOS expression in the brain is found under unstimulated conditions, systemic inflammation induced e.g. by peripheral LPS administration causes a profound induction of iNOS messenger RNA in vascular, glial and neuronal structures of the rat brain. After induction, iNOS is active for a period of 4 to 24 hours and activity typically peaks 6 hours after the LPS injection (Wong et al. 1996).

The ARC and the PVN show strikingly high iNOS induction in response to LPS (Wong et al. 1996). Interestingly, the time-course of iNOS expression in the ARC seems to parallel the time course of LPS-induced anorexia, which is evident about 2 hours after LPS-injection and typically lasts for about 24 hours (Kim et al. 2007). As demonstrated by electrophysiological studies of our group, NO directly inhibits ghrelin-activated neurons of the ARC via the formation of cGMP (Riediger et al. 2006). Therefore, NO modulates neuronal activity of ARC neurons in a way opposite to the well-characterized excitatory effect of ghrelin. This is consistent with an anorectic mode of action. In line with this, our group previously demonstrated that an anorectic dose of peripherally injected LPS attenuated the fasting induced c-Fos expression, a marker for neuronal activity, in the ARC of mice (Becskei et al. 2008). Together these findings support the assumption that LPS inhibits orexigenic ARC neurons via an induction of NO formation. The fact that iNOS deficient mice are more resistant to LPS-induced weight loss and mortality than wild-type mice further supports the pathological role of NO formation under severe inflammation (Wei et al. 1995).

In summary these findings suggest a possible contribution of NO-formation in disease-related anorexia-cachexia; the ARC may be a possible central site mediating this effect.

2.3 The area postrema / nucleus tractus solitarii region

Another brain site which plays an important role in the control of food intake is the area postrema / nucleus tractus solitarii region (AP/NTS). The AP is a highly vascularized circumventricular organ that lacks a blood brain barrier. One of the hormones acting via the AP is the anorectic pancreatic hormone amylin (Lutz 2006). After its secretion into the blood stream, amylin activates AP neurons (Riediger et al. 2001). Ablation of the AP resulted in a significant reduction of the anorectic effect of IP injected amylin in rats (Lutz et al. 1998). For this and other reasons, the AP is considered the primary target site mediating the anorectic effects of amylin.

The NTS is located in close proximity to the AP and receives vagal afferents from the gastrointestinal tract. It is reciprocally connected to the AP via monosynaptic projections (Ferguson 1991). Moreover, the NTS is part of the LPBN – CeA – BNST – axis and projects to several hypothalamic structures, namely the PVN, dorsomedial (DMH) and the ARC nuclei of the hypothalamus and the medial preoptic area (Ricardo and Koh 1978).

It seems possible that the AP/NTS region is also involved in some disease-related symptoms via an NO dependent action. LPS given systemically induces an iNOS-dependent NO production in the NTS which is antagonized by a pretreatment with aminoguanidine, a specific inhibitor of iNOS (Lin et al. 1999). Furthermore, an LPS challenge activates NTS neurons as demonstrated by c-Fos expression (marker for neuronal activation) (Lin et al. 1999). With respect to the LPS-induced anorexia the role of the AP/NTS region is not fully understood. A lesion of the AP/NTS was found to enhance rather than to inhibit the anorectic effect of LPS (Weingarten et al. 1993).

2.4 The JAK-STAT pathway

The Janus kinases and Signal Transducers and Activators of Transcription (JAK-STAT) signaling pathway takes part in the regulation of cellular responses to cytokines and represents an intracellular signaling cascade linking cytokine-mediated receptor activation and gene transcription. The JAK-STAT pathway is stimulated by cytokine receptor activation. The binding of cytokines to the receptor activates the JAKs, which leads to the phosphorylation of the receptor; the STATs bind to the receptor and are phosphorylated as well (pSTAT). Two activated pSTAT molecules dimerize and translocate into the cell nucleus where they modulate gene transcription (Hebenstreit et al. 2005) (Fig. 1).

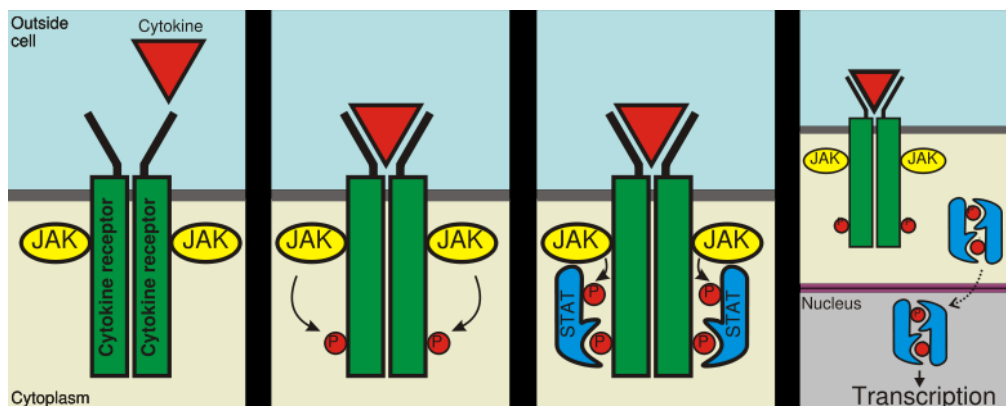


FIG. 1. **Key steps of the JAK-STAT pathway** (figure reproduced with permission from Peter Znamenskiy)

The STAT family comprises at least seven identified transcription factors (STAT1-4, STAT5a/5b and STAT6) (Lim and Cao 2006). Leptin is known to induce STAT3 phosphorylation in the ARC (Hakansson and Meister 1998). It appears plausible that the LPS-induced iNOS expression in the ARC is also upregulated by STAT signaling. Regarding the different STAT isoforms, STAT1 has already been shown to be involved in the regulation of iNOS expression by cytokines (Dell'Albani et al. 2001). Next to the important role of STAT1 for iNOS gene transcription, STAT3 phosphorylation may also be implicated in gangliosides-induced inflammatory responses and in iNOS upregulation in brain microglia cells (Kim et al. 2002). Furthermore it has been demonstrated that intraperitoneal LPS treatment induced STAT3 activation in astrocytes and endothelial cells in rat and guinea pig brains (Rummel et al. 2005).

2.5 NO-based drug therapy

Disease-related anorexia and associated body weight loss is still a major clinical problem that promotes morbidity, mortality and has negative consequences for recovery in many types of diseases. Antagonism of cytokine production and action, and the blockade of prostaglandin synthesis by cyclooxygenase inhibitors represent therapeutic strategies. In view of a possible role of NO in the development of LPS-induced anorexia, a specific blockade of iNOS might also be a promising treatment option.

1400W is a selective and long-acting iNOS-inhibitor showing a very high specificity for iNOS, because it is at least 5000-fold and 200-fold selective for human iNOS versus eNOS and nNOS, respectively, and 1000-fold more potent against rat iNOS than eNOS (Garvey et al. 1997). This specificity minimizes undesired hemodynamic effects that often occur after treatment with less specific NOS inhibitors. Furthermore, the inhibited enzyme did not recover activity after 2h. Thus, 1400W seems to be a long-acting compound that exerts a slowly reversible iNOS inhibition (Garvey et al. 1997). This is also supported by further biochemical in vitro studies showing that 1400W irreversibly inactivates iNOS (Zhu et al. 2004). Importantly, it also seems to act centrally after peripheral application (Czapski et al. 2007). For instance, administration of 1400W improved the histopathological outcome of traumatic brain injury implying a deleterious role for iNOS in this context (Jafarian-Tehrani et al. 2005). Despite these studies

supporting 1400W as a potential therapeutically useful drug, there are currently no studies investigating the effect of specific iNOS blockade in LPS-induced anorexia.

2.6 Aim and Hypothesis

The current study focuses on a possible involvement of NO in disease-related anorexia. This was tested by a pharmacological disruption of iNOS activity using a selective and long acting iNOS inhibitor (1400W). In contrast to the well-established function of NO in the induction of fever, there are currently no studies directly evaluating the involvement of NO in the development of anorexia in disease. We therefore investigated whether peripheral administration of 1400W attenuates LPS-induced anorexia and associated disease symptoms (Fig. 2).

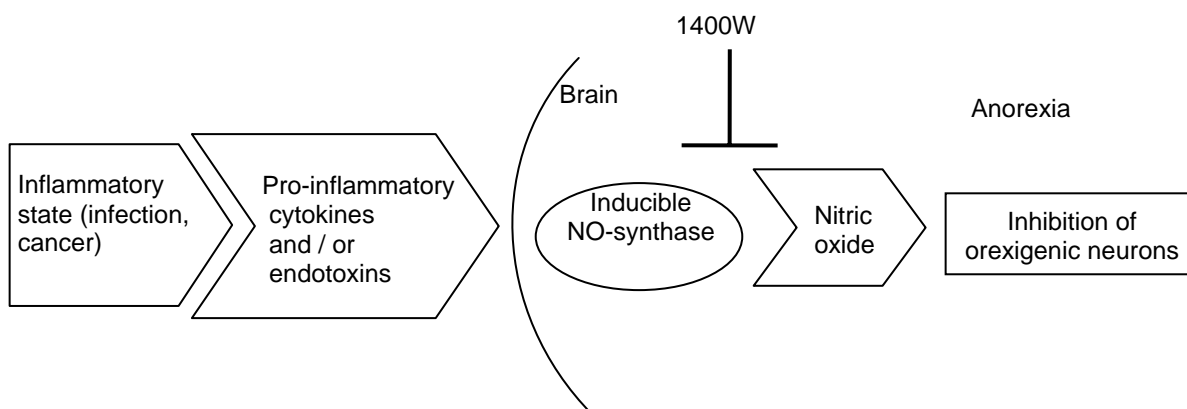


FIG. 2. **Proposed model of NO involvement in disease-related anorexia.** The release of pro-inflammatory cytokines and endotoxins is triggered under inflammatory conditions. In response to these cytokines, the enzyme iNOS is upregulated in the brain, particularly in the arcuate nucleus. This enzyme produces the neuromodulator NO leading to anorexia via an inhibition of orexigenic neurons. A blockade of NO formation by the specific iNOS inhibitor 1400W might therefore attenuate disease-related anorexia.

A further aim of this study was to find evidence for a possible association between LPS-induced anorexia and pSTAT3-formation in the arcuate nucleus and AP/NTS region. We therefore examined the time course of LPS-induced pSTAT3 formation in the ARC and in the AP/NTS region. This was tested by administering LPS and by investigating pSTAT3 formation in the brain at different time points after administration.

Repeated LPS injections result in LPS-tolerance leading to an attenuation of LPS-induced anorexia (Langhans et al. 1993). Endotoxin tolerance was initially described in animals

that survived a lethal dose of bacterial endotoxin if they had been previously treated with a sublethal dose (West and Heagy 2002). In guinea pigs and rats, the attenuation of the febrile response during the development of LPS tolerance is associated with a reduced production of cytokines rather than a decrease in responsiveness to cytokines (Zeisberger and Roth 1998). The biologic significance of the development of tolerance against LPS may be seen in the prevention of damage evoked by excessive cytokine production due to repeated LPS release from bacteria which may lead to a septic shock syndrome and death of the host (Zeisberger and Roth 1998). Based on these studies, we assumed that the well-known attenuation of LPS-anorexia after repeated LPS treatment may be associated with a blunted LPS-induced pSTAT3 formation in the brain.

3 Material and Methods

3.1 Animals and housing

All experiments were carried out using male Wistar rats (Elevage Janvier, Le Genest-St. Isle, France). The animals were individually housed in metabolic cages on a layer of wood shavings or in stainless steel cages with wire mesh floor. The rats were adapted to a 12h/12h light-dark cycle and had free access to standard rodent chow and water. The animals were adapted to their housing conditions and handled for at least 10 days before the onset of the experiments.

All experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland.

3.2 Transmitter surgery

Temperature transmitters were implanted in rats weighting approximately 250g. After the surgery, the rats were transferred to the metabolic cages where they recovered and adapted to the experimental condition for 7 days. During that period, the animals had ad libitum access to standard food and water.

To initiate anesthesia, the animals were placed into an induction chamber containing 5% isoflurane (IsoFlo®; Provet AG, Lyssach, Switzerland). Once the animals reached surgical tolerance, the operation field was shaved and disinfected (Betadine®; Provet AG) and the rats were transferred to a nose cone with reduced isofluran level (set to 2-3%) to maintain anesthesia. To prevent the animals from corneal desiccation, an ophthalmic ointment (Vitamin-A Dispersa®, CIBA Vision AG, Niederwangen, Switzerland) was applied. After a 1.5 – 2 cm long midline incision in the skin with a scalpel, the muscle tissue was reached and opened along the *linea alba* with surgical scissors. The sterile transmitters (see 3.6) were placed into the abdominal cavity. Then, the abdominal muscle wall was sutured (absorbable suture, Vicryl 3-0; Ethicon, Norderstedt, Germany) along the *linea alba*; the skin was closed with wound clamps (suture clips, B. Braun Medical AG, Sales Support Aesculap, Sempach, Switzerland).

After the surgery, the rats received subcutaneous injections of enrofloxacin (10mg/kg; Baytril®; Provet AG) for antibiotic and flunixin (0.01 mg/kg; Finadyne®; Provet AG) for analgesic prophylaxis. Furthermore, a prophylactic 5ml depot of 0.9% NaCl (Fresenius

Kabi AG) was injected subcutaneously (s.c.) to avoid postsurgical dehydration. The rats were then kept under a heating lamp until fully awake.

3.3 Metabolic cages (PhysioScan System)

Sixteen metabolic cages in an open-circuit indirect calorimetry system were used (AccuScan Instruments Inc., Columbus, OH, USA) to measure food and water intake, body temperature, activity, energy expenditure and the respiratory quotient. The plexiglas cages had a size of 42cm x 42cm x 30cm and were closed with air-tight lids. Ambient air was pumped through the cage via a manually adjustable flow controller (flow rate set to 2l/min). Air entering and leaving each cage was monitored for its O₂ and CO₂ content by zirconia. The cages were connected to two O₂/CO₂-analyzers (8 cages per analyzer) to evaluate the outcoming air of each cage for a period of 30s every 5 minutes. This allowed to calculate various parameters, which were saved to the connected PC using the manufacturer's software (PhysioPlot Version 1.80, Integra ME Version 2.21; AccuScan Instruments Inc.):

Volume of O₂ consumed:

$$VO_2 \text{ [ml/kg/min]} = \frac{(\text{Flow [ml/min]}) \times (V_1 + V_2)}{100 \times (\%N_2 \text{ Ref}) \times (\text{Weight [kg]})}$$

$$\%N_2 \text{ Ref} = 100 - ((\%O_2 \text{ Ref}) + (\%CO_2 \text{ Ref}))$$

$$V_1 = (\%N_2 \text{ Ref}) \times (\%O_2 \text{ Change})$$

$$V_2 = (\%O_2 \text{ Ref}) \times ((\%O_2 \text{ Change}) - (\%CO_2 \text{ Change}))$$

Ref = room air

Change = difference in the gas content between room air and cage air

Volume of CO₂ produced:

$$VCO_2 \text{ [ml/kg/min]} = \frac{(\text{Flow [ml/min]}) \times (\%CO_2 \text{ Change})}{(\text{Weight [kg]} \times 100)}$$

Respiratory quotient:

$$RQ = \frac{VCO_2}{VO_2}$$

Energy expenditure was calculated according to Weir (Weir, 1949) using the following equation, with O₂ consumption and CO₂ production normalized for body weight:

$$EE \text{ [kcal/kg/min]} = \frac{(3.9 \times VO_2) + (1.1 \times VCO_2)}{1000}$$

3.4 Diets

The rats had ad libitum access to standard laboratory rat chow and water. Chow was either pelleted for the pSTAT3 experiments (GLP 3433; Provimi Kliba AG, Kaiseraugst, Switzerland) or powdered for the experiment conducted in metabolic cages (GLP 3433-9.24; Provimi Kliba AG). This diet was chosen to avoid food hoarding and to be able to detect small changes in food consumption. For food composition of both diets see table 1.

TABLE 1: **Food composition of pelleted and powder chow** (according to the manufacture Provimi Kliba; GLP 3433 and GLP 3433-9.24)

Dry matter	88 %
Protein	18.5 %
Fiber	4.5 %
Fat	4.5 %
Ash	6.3 %
Nitrogen-free extract (NFE)	54.2 %
Starch	35 %
<hr/>	
Metabolizable energy	13.2 MJ/kg

3.5 Food and water consumption

Water bottles and food cups were placed on scales to measure water and food intake

continuously. The measurements were saved on the computer in 5min intervals and were used to calculate cumulative food intake and water consumption.

3.6 Body temperature and physical activity measurements (DSI Telemetry System)

For body temperature and activity measurements, we implanted DSI PhysioTel® TA-F40 Small Animal Transmitters (Data Sciences International, St. Paul, MN, USA) into the peritoneal cavity of each animal. The receiver plates were placed underneath the cage. From these plates, the data were forwarded to the connected PC, where they were stored and evaluated. Recordings were saved every five minutes and processed using the included software (Acquisition Version 4.00, Analysis Version 4.00; Dataquest A.R.T.TM). The activity data represent a semi-quantitative measure of locomotor activity. In other words, a high activity count means that the animal was active, whereas a low activity count means the animal was less active.

3.7 Perfusion and imunohistochemistry

For the immunohistochemical detection of pSTAT3, rats were deeply anesthetized at different time points after LPS or 1400W/LPS administration by an IP-injection of pentobarbital (Nembutal; Abbott Laboratories, Chicago, IL, USA). After reaching surgical tolerance, the thorax was opened and the animals were transcardially perfused with 0.9% NaCl (room temperature, 1.5 min) followed by ice cold paraformaldehyd solution (2% PFA in phosphatbuffer solution 0,1M see table 2; 2 min). After removing the brains, they were postfixed in 2% PFA for 1 hour at 4°C. The brains were transferred to 0.02 M KPBS containing 20% sucrose for the following 48 hours at 4°C. After this cryoprotection, the brains were snap frozen using CO₂ gas (exposure time approx. 1 min). Coronal sections (20µm) were cut in a cryosectioning system (Leica CM3050 S, Leica Microsystems, Nussloch, Germany), thaw-mounted on microscopic glass slides (Super Frost Plus slides; Faust, Schaffhausen, Switzerland) and stored at -20°C until further processing.

stock solution	substances	concentration [g/l]	end concentration in PB [M]
I	NaH ₂ PO ₄ •H ₂ O	27,8	0,1
II	Na ₂ HPO ₄ •2H ₂ O	35,6	0,1

TABLE 2: **Composition of used stock solutions for 0,1M phosphate buffer.** The phosphate buffer contained 190ml of solution I and 810ml of solution II, diluted 1:1 with dest. water and adjusted to a pH of 7,2. 40g PFA were dissolved in 1l distilled water and diluted 1:1 with 0,2 M PB to final PFA concentration of 2%.

To detect pSTAT3, the sections were air-dried at room temperature (1h) and rehydrated in 0.02 M KPBS (see table 3). Afterwards the sections were treated with 0.3% NaOH + 0.3% H₂O₂ (20 min), followed by 0.3% glycine (10 min) and 0.03% sodium dodecyl sulfate (SDS, 10 min; all in 0.02M KPBS). Unspecific binding was blocked by 20 min incubation in blocking solution (KPBS containing 4% normal donkey serum, 0.4% Triton X-100, 1% bovine serum albumin). The primary antibody (rabbit anti-pSTAT3 1:500; Cell signaling; in blocking solution) was applied for 48 hours at 4° C, followed by the secondary antibody (donkey anti-rabbit Alexa-555 1:100; Jackson ImmunoResearch) for 2h at room temperature. Finally, the sections were coverslipped with citifluor (Citifluor Ltd).

substances	concentration (g/l)
KH ₂ PO ₄	4,85
K ₂ HPO ₄	28,35
NaCl	89

TABLE 3: **Composition of stock solution for 0.02 M KPBS.** Before use, the solution was diluted 1:9 with dest. water

3.8 Experimental paradigms

3.8.1 Effect of 1400W on LPS-induced anorexia and associated disease symptoms

To investigate a possible involvement of NO in LPS induced anorexia, feeding studies with rats in metabolic cages were conducted. LPS (Escherichia coli O111:B4, L2630-25MG, Sigma Aldrich Chemie GmbH, Buchs, Switzerland) and 1400W (100050-5, VWR International AG, Dietikon, Switzerland) were diluted in sterile 0,9% NaCl (Fresenius Kabi AG, Stans, Switzerland). The rats were divided into four groups

consisting of four animals each, including a saline control group, a LPS treated group, a 1400W-treated control group and a group which was injected with LPS and 1400W. LPS (100µg/kg; i.p.), 1400W (10mg/kg; s.c.) or saline were injected shortly before dark onset. Effective doses of 1400W used in vivo range between 5mg/kg and 20mg/kg (Parmentier et al. 1999; Jafarian-Tehrani et al. 2005). Therefore a dose of 10mg/kg was chosen for the current study. For the injections, sterile syringes (Omnifix 1 ml, Braun Melsungen AG, Melsungen, Germany) and sterile needles (Terumo, 26G, Leuven, Belgium) were used. Gas exchange (to calculate EE and RQ), body temperature, activity, food and water intake were measured during 23 hours after injections.

This study was performed using a crossover design between the saline treated and the LPS-injected animals, and between the 1400W and LPS/1400W treated animals with 12 days recovery time between the two trials. Hence, each rat received only one LPS treatment to avoid the development of LPS tolerance (see 2.6).

3.8.2 Time course of LPS-induced pSTAT3 formation

The aim of this study was to investigate the time course of LPS-induced pSTAT3-formation in the ARC and AP/NTS and to establish an adequate timepoint for pSTAT3 to be considered for the subsequent studies under our experimental conditions (see 3.8.3).

Animals weighting approximately 300g were injected with either LPS (100µg/kg; i.p.) or saline at dark onset. Two hours later half of the LPS and saline treated rats were anesthetized and perfused transcardially (see 3.7); the remaining animals were perfused 4 hours after treatment. In total 26 animals were divided into 4 groups (n = 5-10/group). Brains were frozen, sliced and stained according the protocol described above. The stained slices were evaluated using a fluorescent microscope by counting the number of pSTAT3 positive cells manually in every second slice of the analyzed brain regions in a blind fashion.

3.8.3 Temporal association between pSTAT3 formation and LPS-induced anorexia

It had already been shown that repeated LPS injections result in LPS-tolerance, leading e.g. to an attenuation of LPS-induced anorexia (Langhans et al. 1993). Based on these studies we assumed that the well-known attenuation of LPS-anorexia after repeated LPS treatment may be associated with a blunted LPS-induced pSTAT3 formation in the brain. For this study 20 rats were used weighting approximately 200g at the beginning of the

experiment. For the feeding trial the animals were subdivided into a saline (n=13) and a LPS (n=7) treated group. On day one of the experiment the rats were injected at dark onset either with saline or LPS. Food intake was measured in external food hoppers 4, 6 and 12 hours after the treatment. After one day of recovery (day 2), the same procedure was performed on day 3 to simulate LPS-tolerance. On day 5, the saline treated group was divided into one saline group (control; n=6), and one group of rats receiving a single LPS injection (LPS; n=7). The rats that had received LPS on the previous days received a third LPS treatment (desensitization group; n=7, see table 4). Four hours after the last injections all animal were anesthetized and perfused transcardially. Brains were frozen, sliced and processed for pSTAT3 immunoreactivity. The perfusion time point was chosen according to the outcome of the experiment described above (3.8.2), showing a significant increase in pSTAT3 positive cells 4 hours after LPS injection in the ARC and the AP/NTS region.

	day 1	day 3	day 5
control group (saline)	saline	saline	saline
LPS group (1xLPS, 100µg/kg)	saline	saline	LPS
desensitization group (3xLPS, 100µg/kg)	LPS	LPS	LPS

TABLE 4: Desensitization paradigm

3.9 Statistical analyses

For statistical evaluation of experiments conducted in metabolic cages, differences in the LPS treated groups relative to their control groups at each time point were calculated. These values were then compared using Student's *t*-test. In both the feeding and immunohistological studies, the effects of LPS were compared to saline injection by Student's *t*-test at the different time points separately. When data were not normally distributed a non-parametric Mann-Whitney rank sum test was used. The immunohistological slides from the study investigating an association between pSTAT3 formation and LPS-induced anorexia were analyzed using one-way ANOVA to compare the different treatment groups. In the immunohistological studies the mean value of the cell count/section of an individual animal was used for statistical analyses. All data are presented as means \pm SEM. $p < 0.05$ was considered significant.

4 Results

4.1 Effect of 1400W on LPS-induced anorexia and associated disease symptoms

In this study the effect of specific iNOS inhibition by 1400W on LPS-induced anorexia was investigated. In addition, several other parameters (water intake, body temperature, activity, EE, RQ) were also recorded and analyzed. LPS (100 μ g/kg; i.p. [n=8]), 1400W (10mg/kg; s.c. [n=8]), LPS in combination with 1400W (n=8) or saline (control [n=8]) were injected.

1400W partly reversed the LPS-induced reduction of food intake compared to the LPS group (Fig. 3A). LPS decreased eating from about 2 hours after injection and food intake remained significantly decreased for 23 hours ($t = 23$ h; saline: 26.45 ± 4.8 g, LPS: 13.93 ± 2.6 , paired t-test, $p < 0.001$). The inhibitor 1400W alone slightly reduced food intake relative to the saline control group. However, this difference was not significant at the end of the measurement period ($t = 23$ h; saline: 26.45 ± 4.8 , 1400W: 23.76 ± 1.6 , paired t-test, $p = 0.22$). For statistical evaluation we then compared the differences in the LPS-treated groups relative to their representative control groups (LPS vs. saline and LPS/1400W vs. 1400W). Figure 3B shows the differences for each time point. The LPS-induced reduction in eating of 1400W treated animals was significantly smaller than in saline-treated animals between 8 and 23 hours after LPS injection (see Fig. 3B for individual time points).

Similar to the effects on food intake, the LPS-induced reduction of water intake was attenuated by treatment with 1400W (Fig. 4A). The LPS-induced reduction of water intake in 1400W-treated animals was significantly less between 5 and 23 hours than in rats not receiving 1400W (see Fig. 4B for individual time points).

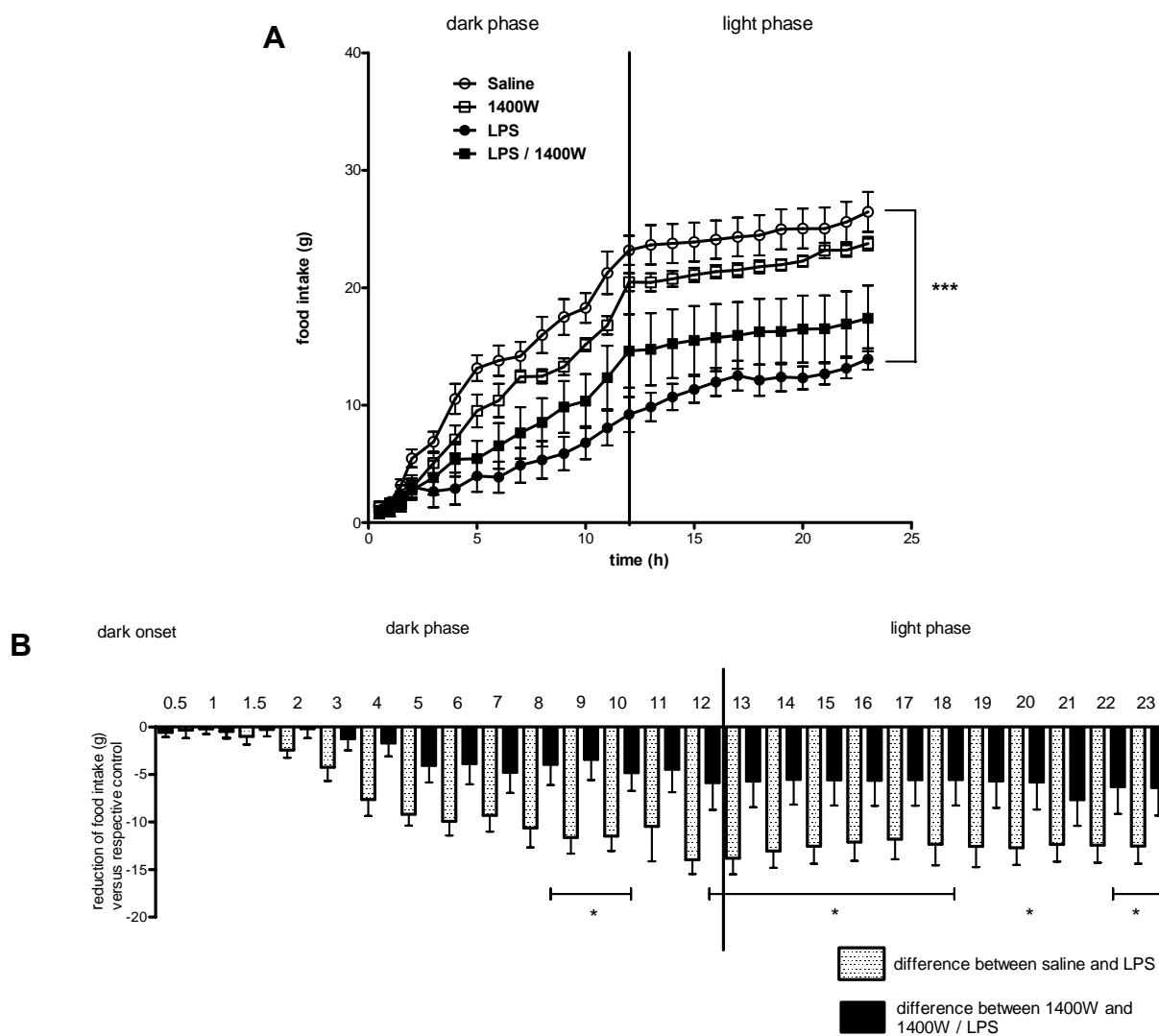


FIG. 3. The iNOS inhibitor 1400W attenuated the LPS-induced suppression of food intake. The figure shows a 23h measurement period of cumulative food intake (A). Rats were injected in a crossover design for LPS treatment (n=8) before dark onset. LPS significantly reduced food intake compared to saline control group at time point 23h after treatment. (B) Differences in the LPS-treated groups relative to their control groups at the respective time points. Data are expressed as mean \pm SE (* $p < 0.05$).

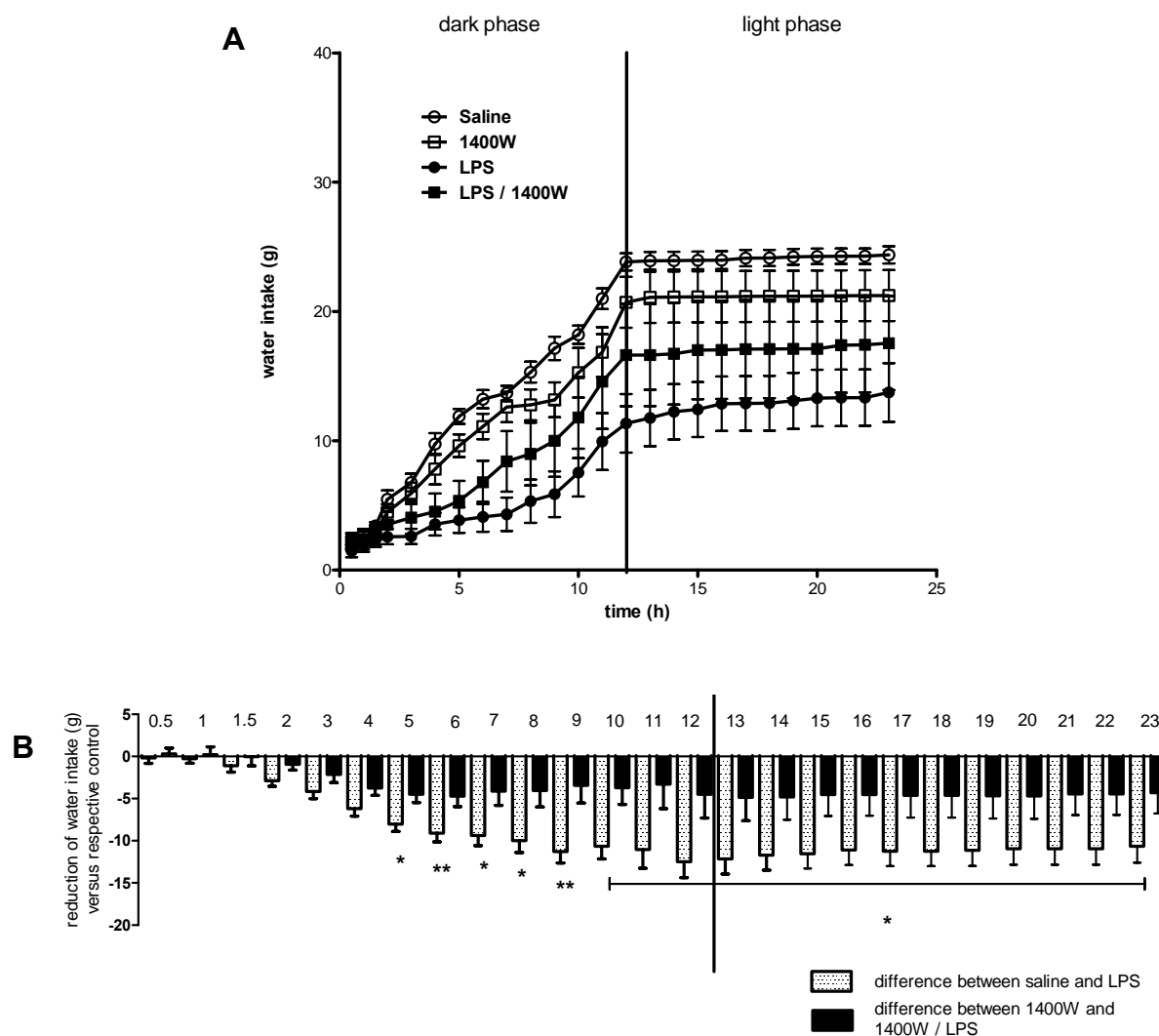


FIG. 4. The iNOS inhibitor 1400W attenuated the LPS-induced reduction of water intake. The figure shows a 23h measurement period of cumulative water intake (A). Rats were injected in a crossover design for LPS treatment (n=8) before dark onset. (B) Differences in the LPS-treated groups relative to their control groups at the respective time points. Data are expressed as mean \pm SE (* $p<0.05$, ** $p<0.01$).

The LPS-induced fever response was also partly prevented by 1400W treatment (Fig. 5A). As shown by the statistical analyses (Fig. 5B), LPS induced a stronger effect in the saline-treated group than in the 1400W-treated group at various time points between 2 and 22 hours after injections (see Fig. 5B for individual time points).

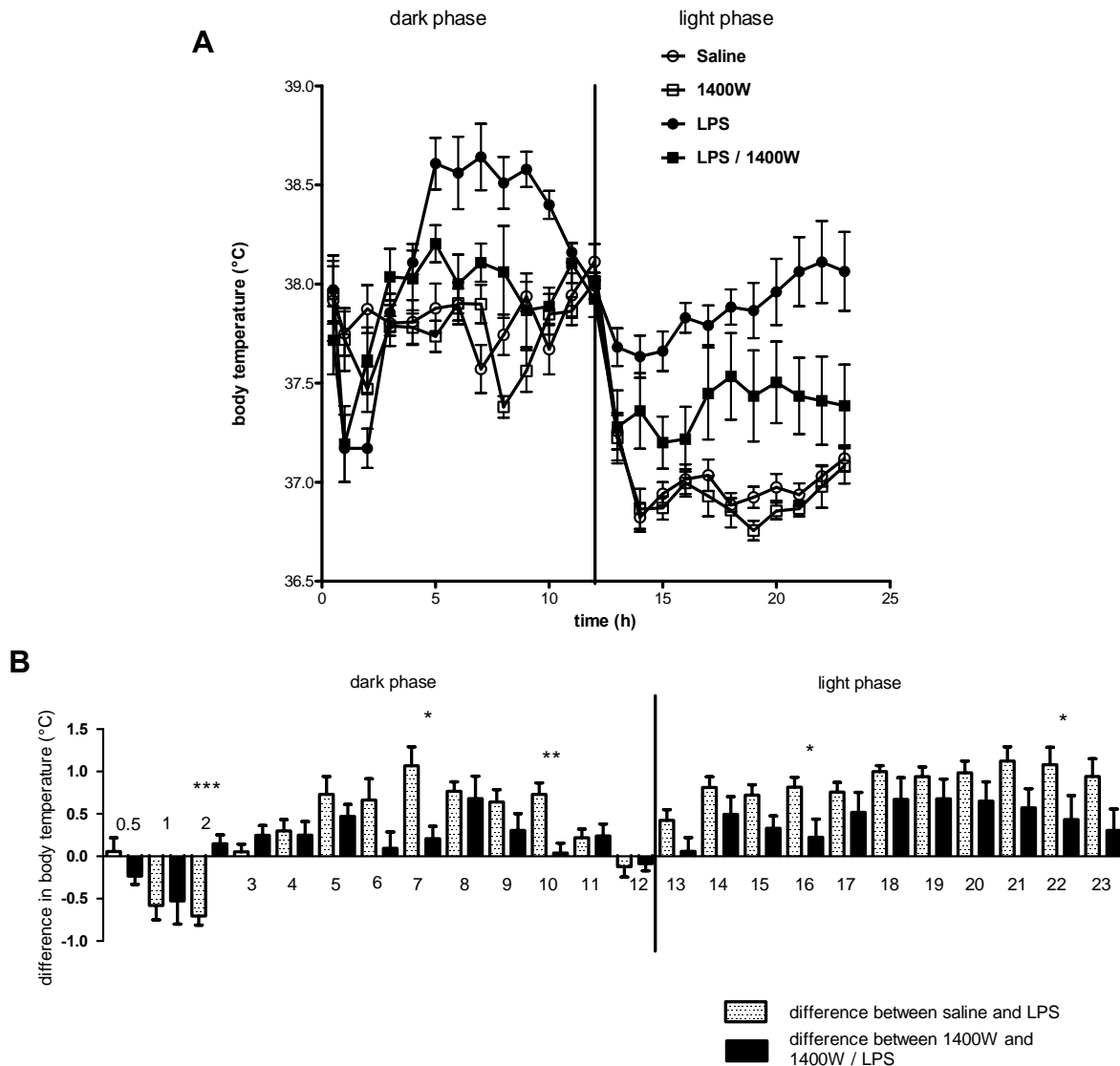


FIG. 5. The iNOS inhibitor 1400W attenuated the LPS-induced fever response. The figure shows a 23h measurement period of body temperature (A). Rats were injected in a crossover design for LPS treatment (n=8) before dark onset. (B) Differences in the LPS-treated groups relative to their control groups at the respective time points. Data are expressed as mean \pm SE (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

LPS reduced locomotor activity in the dark phase; this was partly counteracted by 1400W because 1400W/LPS animals showed a higher activity than the LPS-control group (Fig. 6A). 1400W control animals exhibited a similar locomotor activity as saline-treated animals. The LPS-induced reduction of activity in 1400W-treated rats was significantly smaller at various time points between 6 and 17 hours after injections (see Fig. 6B for individual time points). A quantification of the differences in cumulative locomotor activity between the LPS-treated groups and their respective control groups (LPS vs. saline and LPS/1400W vs. 1400W) is shown in figure 6C. Compared to controls, the LPS-induced

reduction in cumulative activity of 1400W treated animals was significantly smaller from 8 to 23 hours after injections (Fig. 6C).

A further effect of LPS was a pronounced decrease in energy expenditure (EE), particularly during the dark phase (Fig. 7A). The LPS-induced reduction of EE in 1400W-treated rats was significantly attenuated at various time points between 8 and 12 hours after injections (see Fig. 7B for individual time points). The difference in cumulative EE between the LPS-treated groups and their respective control groups was not significant, but was close to significance at time point 12h [$p = 0.0520$], (Fig. 7C)].

LPS-treated animals had a lower respiratory quotient relative to both saline and 1400W treated rats; this indicates a shift towards increased lipid metabolism (Fig. 8A). 1400W did not affect the LPS-mediated reduction in RQ (Fig. 8B).

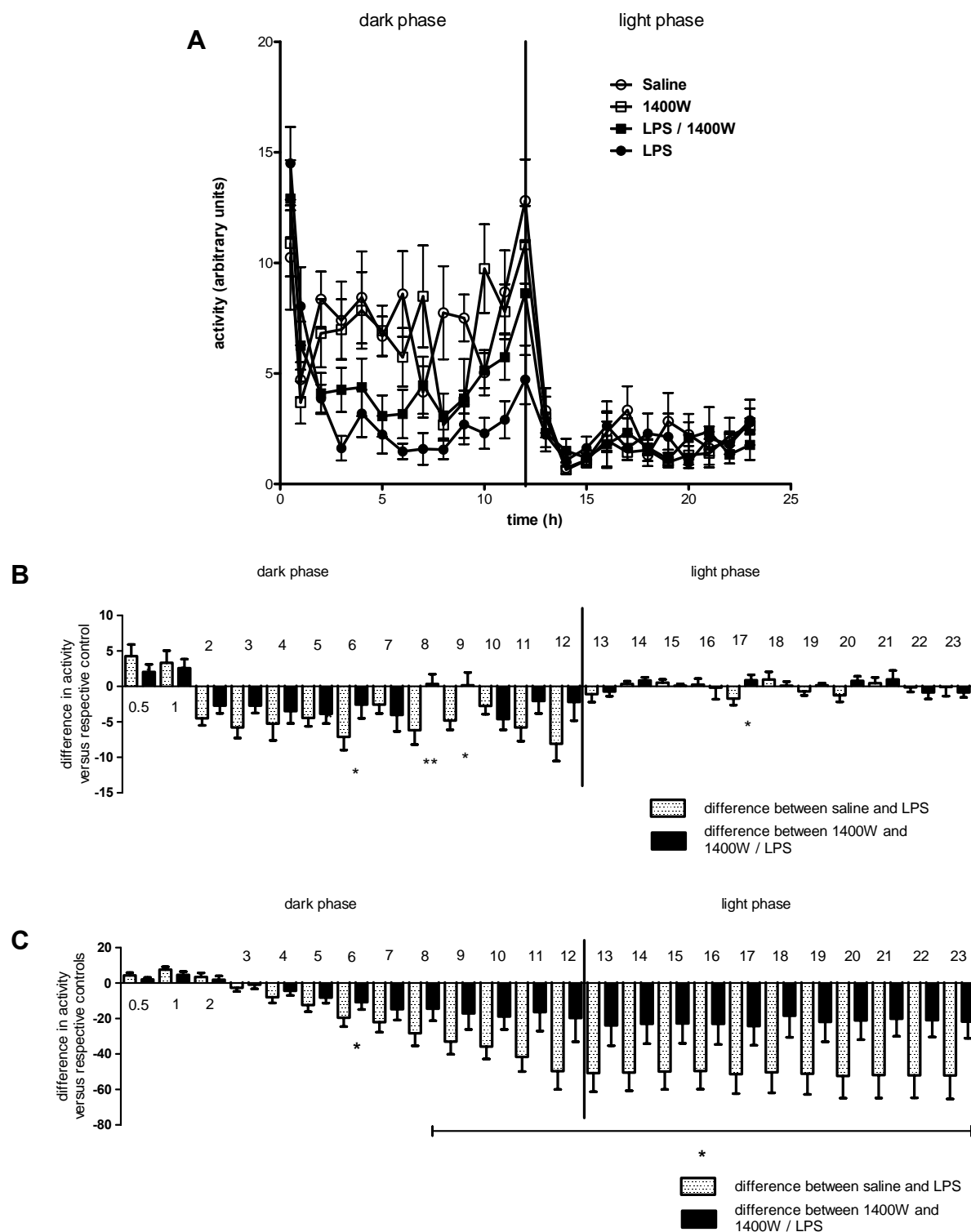


FIG. 6. The iNOS inhibitor 1400W attenuated the LPS-induced suppression of activity. The figure shows a 23h measurement interval of physical activity (A). Rats were injected in a crossover design for LPS treatment (n=8) before dark onset. (B) Differences in the LPS-treated groups relative to their control groups at the respective time points. (C) Cumulative differences in the LPS-treated groups relative to their control groups. Data are expressed as mean \pm SE (* $p < 0.05$, ** $p < 0.01$).

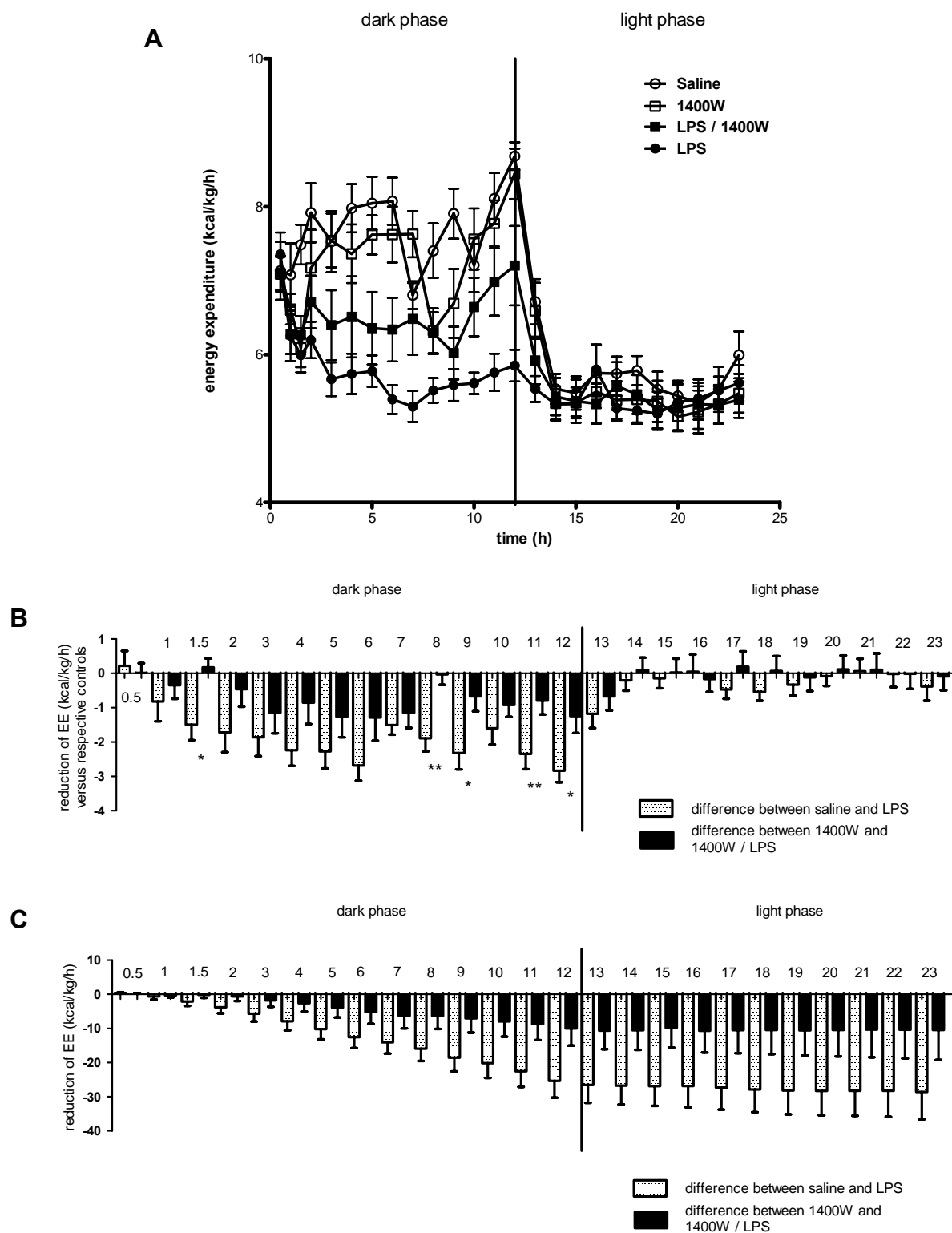


FIG. 7. The iNOS inhibitor 1400W attenuated the LPS-induced reduction of energy expenditure. The figure shows a 23h measurement period of energy expenditure (A). Rats were injected in a crossover design for LPS treatment (n=8) before dark onset. (B) Differences in the

Results

LPS-treated groups relative to their control groups at the respective time points. (C) Cumulative differences in the LPS-treated groups relative to their control groups. Data are expressed as mean \pm SE (* $p < 0.05$, ** $p < 0.01$).

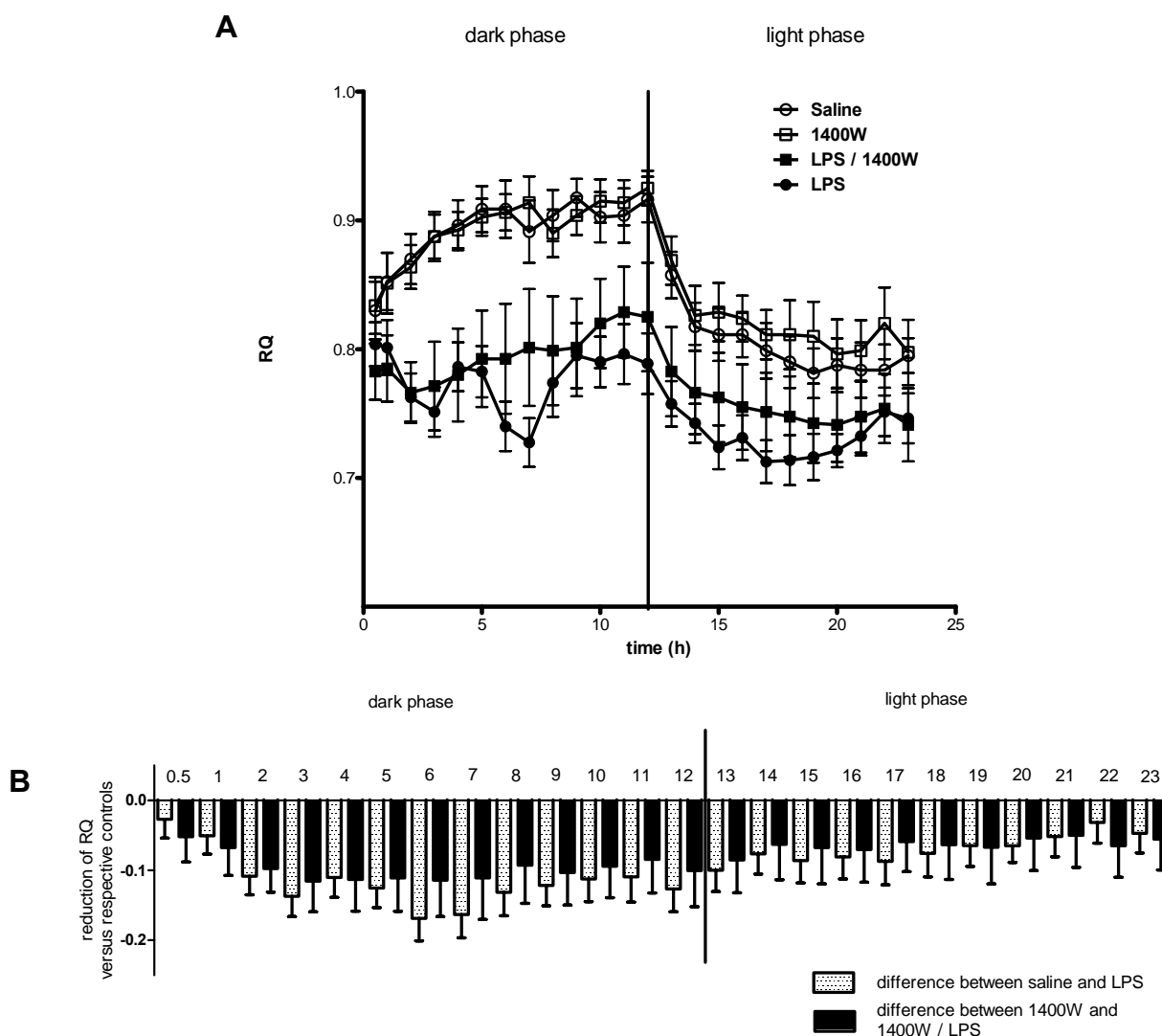


FIG. 8. The iNOS inhibitor 1400W did not attenuate the LPS-induced decline of respiratory quotient. The figure shows a 23h measurement period of respiratory quotient (A). Rats were injected in a crossover design (n=8) before dark onset. (B) Differences in the LPS-treated groups relative to their control groups at the respective time points. There was no statistically difference at any time point.

4.2 Time course of LPS-induced pSTAT3 formation

As the second part of this project, we carried out an immunofluorescence staining for pSTAT3 to find evidence for a temporal association between LPS-induced anorexia and pSTAT3-formation in the ARC and AP/NTS region. Ad libitum fed rats were treated i.p. with 100µg/kg LPS (n=15) or saline (n=11) at dark onset and perfused 2 or 4 hours after the injections. The ARC and the AP/NTS region were processed for pSTAT3 immunoreactivity as described (3.7).

While we observed a small but significant reduction in the number of pSTAT3 positive cells 2h after LPS treatment, LPS induced a strong increase in the number of pSTAT3 immunoreactive cells 4 h after injection in the arcuate nucleus (Fig. 9A). Representative immunostainings of all experimental groups are shown in Figure 9B.

Similar to the ARC, LPS induced a pronounced pSTAT3 formation in the AP/NTS region 4 hours after administration (Fig. 10A+B). Representative immunostainings of all experimental groups are shown in Figure 10C.

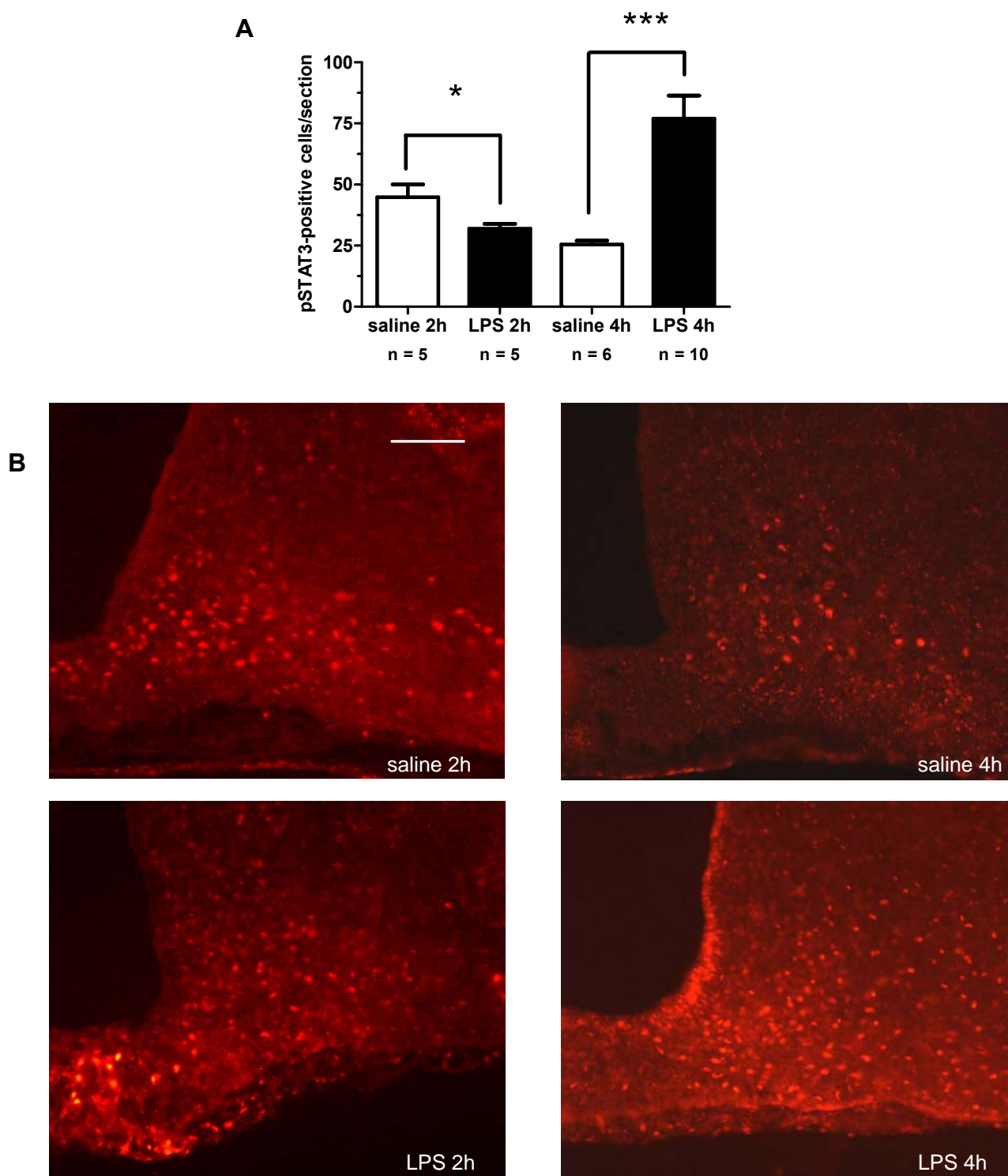


FIG. 9. Effect of LPS-injection on pSTAT3 formation in the arcuate nucleus. After 2 hours LPS significantly reduced pSTAT3 formation, while after 4 hours LPS led to a significant increase in the number of pSTAT3 positive cells compared to the saline control group in the ARC (A). Data are expressed as mean \pm SE. (* $p < 0.05$, *** $p < 0.001$). Representative photomicrographs illustrating the effect of systemic injections of LPS (100 $\mu\text{g/kg}$ IP) on the expression of pSTAT3 in the ARC (B). Scale bar: 100 μm .

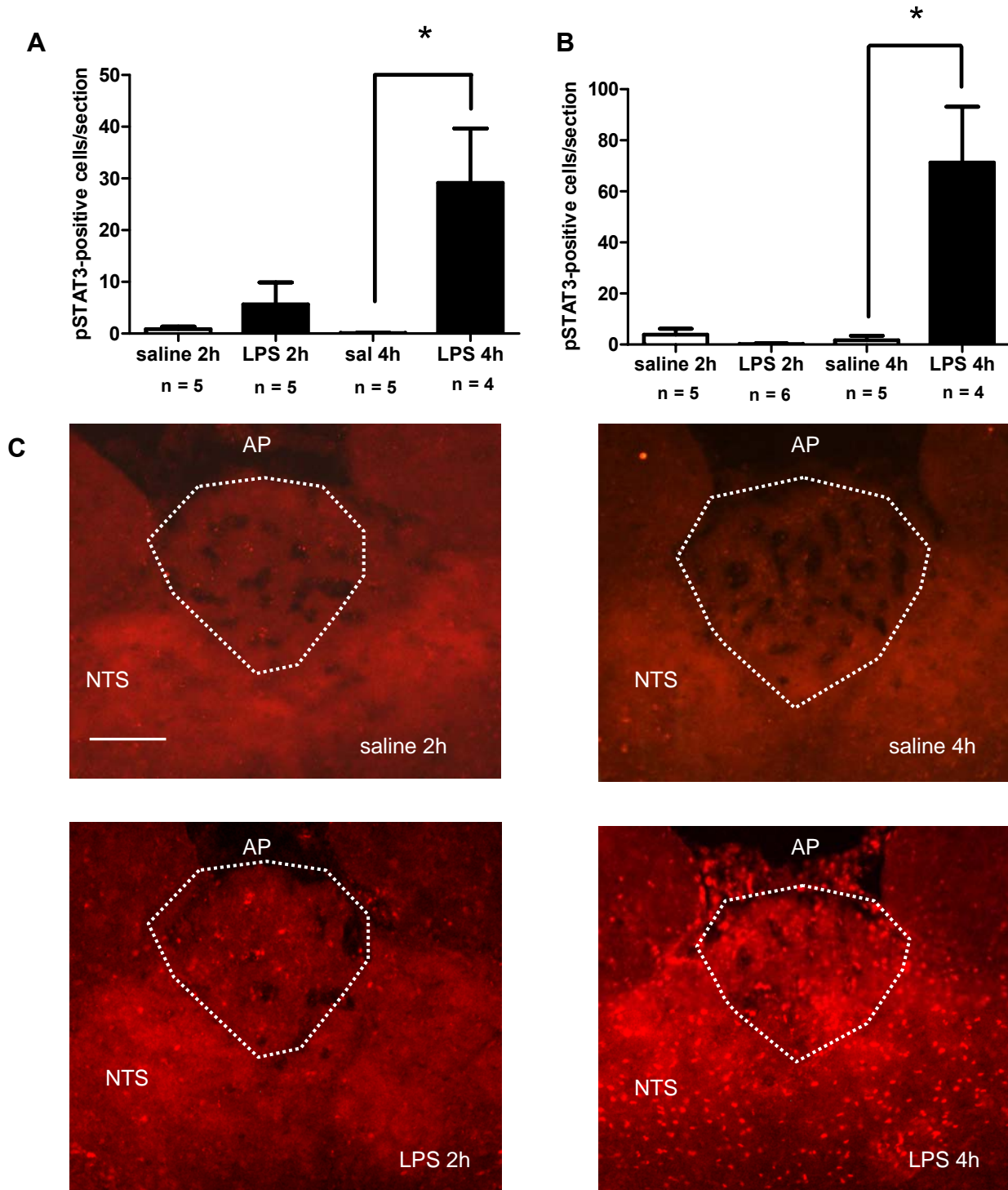


FIG. 10. Effect of LPS-injection on pSTAT3 formation in the area postrema/nucleus tractus solitarii region. LPS (4h) induced a significantly higher number of pSTAT3 positive cells compared to the saline-treated control group in the AP (A) and NTS (B). Data are expressed as mean \pm SE. * $p < 0.05$. Representative photomicrographs illustrating the effect of systemic injections of LPS (100 μ g/kg IP) on the expression of pSTAT3 in the AP/NTS-region (C). AP = area postrema. NTS = nucleus tractus solitarii. Scale bar: 100 μ m.

The pSTAT3 immunoreactive structures observed 4h after LPS treatment appeared to have a different size and shape than cells that were observed in the saline-treated groups. While under control conditions pSTAT3 immunoreactivity seemed to be cytoplasmatic, the signal appeared to have a nuclear location, possibly suggesting an increased nuclear translocation of pSTAT3 in response to LPS (Fig. 11A/B).

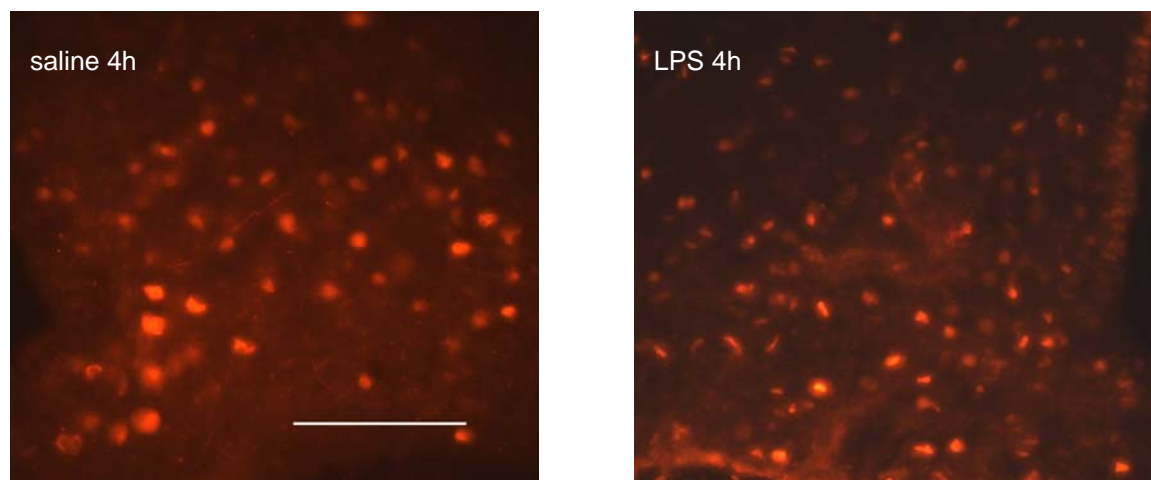


Fig. 11. Photomicrographs demonstrating pSTAT3 formation in the ARC at higher magnification. The rats received either saline (A) or LPS (100 μ g/kg IP) (B). After LPS treatment, pSTAT immunoreactive staining were smaller and had a more elongate shape compared to the cells in the saline treated group, which was larger and more roundish. Scale bar: 100 μ m.

4.3 Temporal association between pSTAT formation and LPS-induced anorexia

Based on the previous experiment, a follow-up study with repeated LPS treatments was performed to investigate a possible temporal association between pSTAT3 formation and LPS-induced anorexia. On day one, rats were given a single i.p. injection of LPS (100 μ g/kg; n=7) or saline (n=13) at dark onset and food intake was measured manually 4, 6 and 12 hours after injections. Food intake of LPS-treated animals was significantly reduced 6 and 12 hours after the injections (Fig. 12A). On day three of the experiment, the same procedure was repeated. In contrast to the first treatment day, there was no significant LPS-induced reduction of eating at any time point; this indicates at least partial LPS desensitization (Fig.12B).

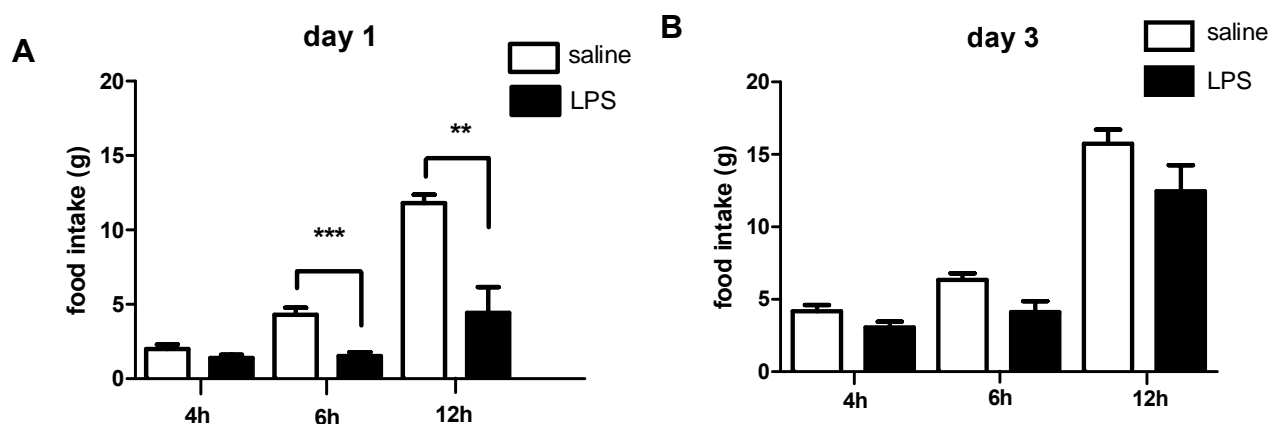


Fig. 12. **Effect of repeated injections of LPS (100 μ g/kg) on food intake.** Single LPS injection (n=7) resulted in significantly reduced food intake in contrast to saline injection (n=13) (A), whereas no anorectic response to LPS was observed when the same animals received LPS again on day 3 (n=7) (B). Data are expressed as mean \pm SE. (**p<0. 01, ***p<0.001).

On day 5, the animals were treated for a third time with LPS or saline for the detection of pSTAT3 immunoreactivity. In order to include an LPS sensitive positive control group that received only a single LPS treatment, half of the animals that had received saline on days 1 and 3 were now injected with LPS. 4 hours after the last treatments, the rats were transcardially perfused.

Similar to the results presented above, single LPS treatment induced a significant increase in the number of pSTAT3 positive cells in all investigated brain areas (Fig. 13-15). In contrast to the single LPS treatment, animals that had received repeated LPS injections showed no LPS-induced pSTAT formation 4h after the last injection in the ARC compared to saline-treated animals (Fig.13). There was a similar outcome in the AP and the NTS (Fig. 14 and 15); only rats after a single LPS treatment showed a significantly higher number of pSTAT3 positive cells than the saline controls.

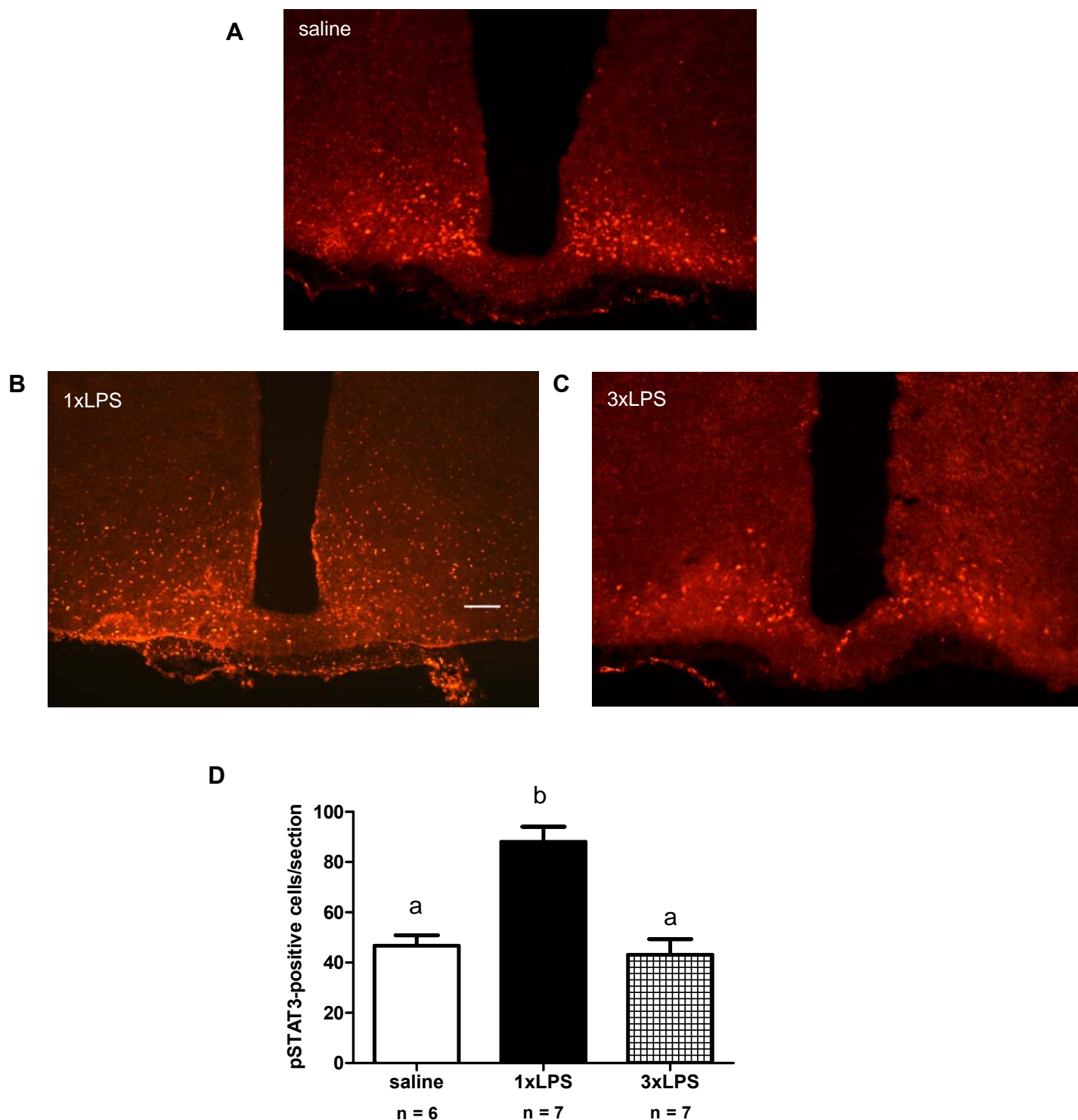


FIG. 13. Effect of repeated injections of LPS (100 μ g/kg) on pSTAT3 formation in the arcuate nucleus. The rats received either saline (A; saline/saline/saline), single (B; saline/saline/LPS) or three LPS (100 μ g/kg IP) injections (C; LPS/LPS/LPS) at dark onset on days 1, 3 and 5. Single LPS treatment induced a significantly higher number of pSTAT3 positive cells than saline; no effect was observed after repeated LPS treatment (D). Scale bar: 100 μ m. Different letters mean statistically significant values between groups.

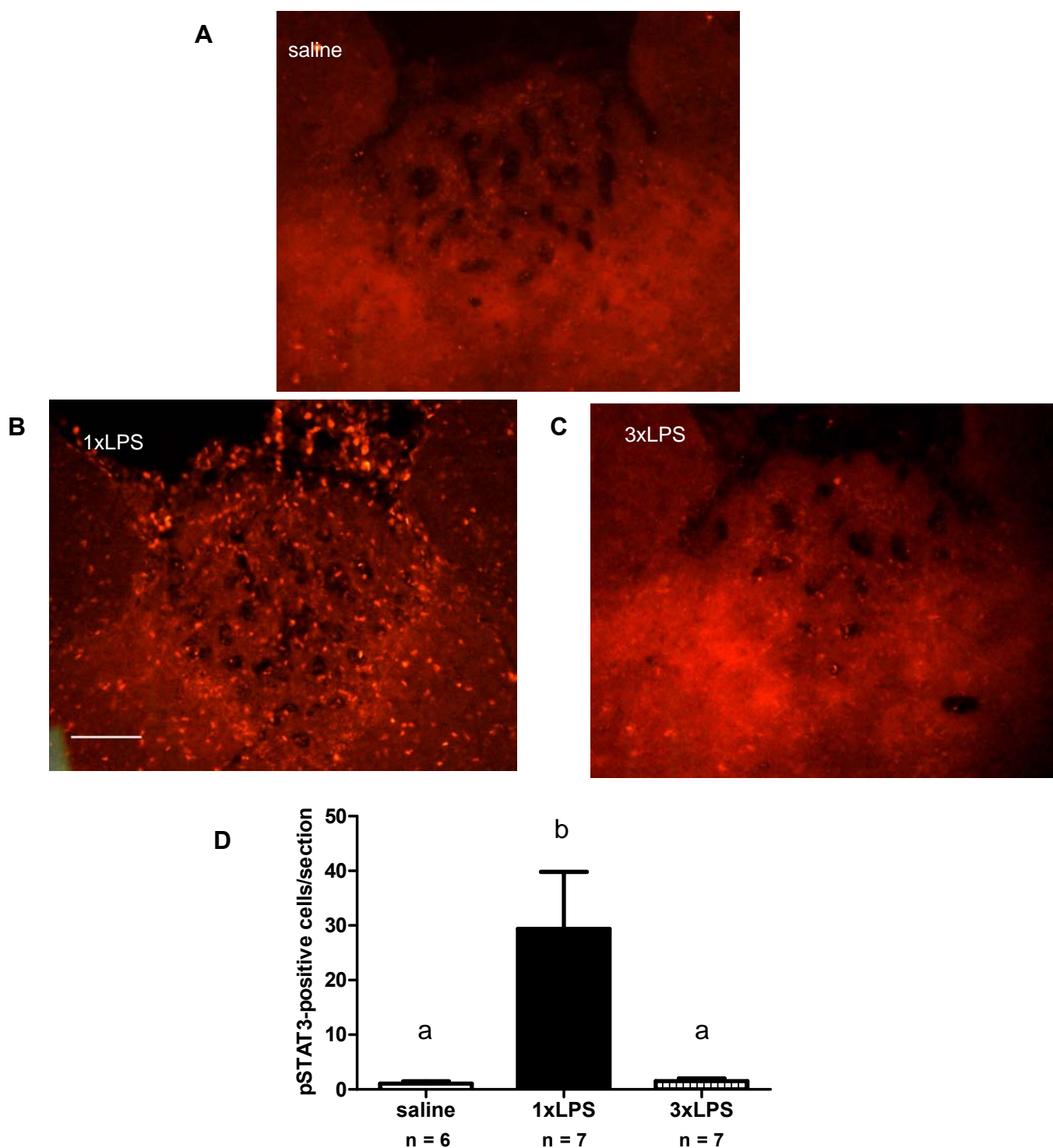


FIG. 14. **Effect of repeated injections of LPS (100µg/kg) on pSTAT3 formation in the AP.** The rats received either saline (A; saline/saline/saline), single (B; saline/saline/LPS) or three LPS (100µg/kg IP) injections (C; LPS/LPS/LPS) at dark onset on days 1, 3 and 5. Single LPS treatment induced a significantly higher number of pSTAT3 positive cells than saline; no effect was observed after repeated LPS treatment (D). Scale bar: 100µm. Different letters mean statistically significant values between groups.

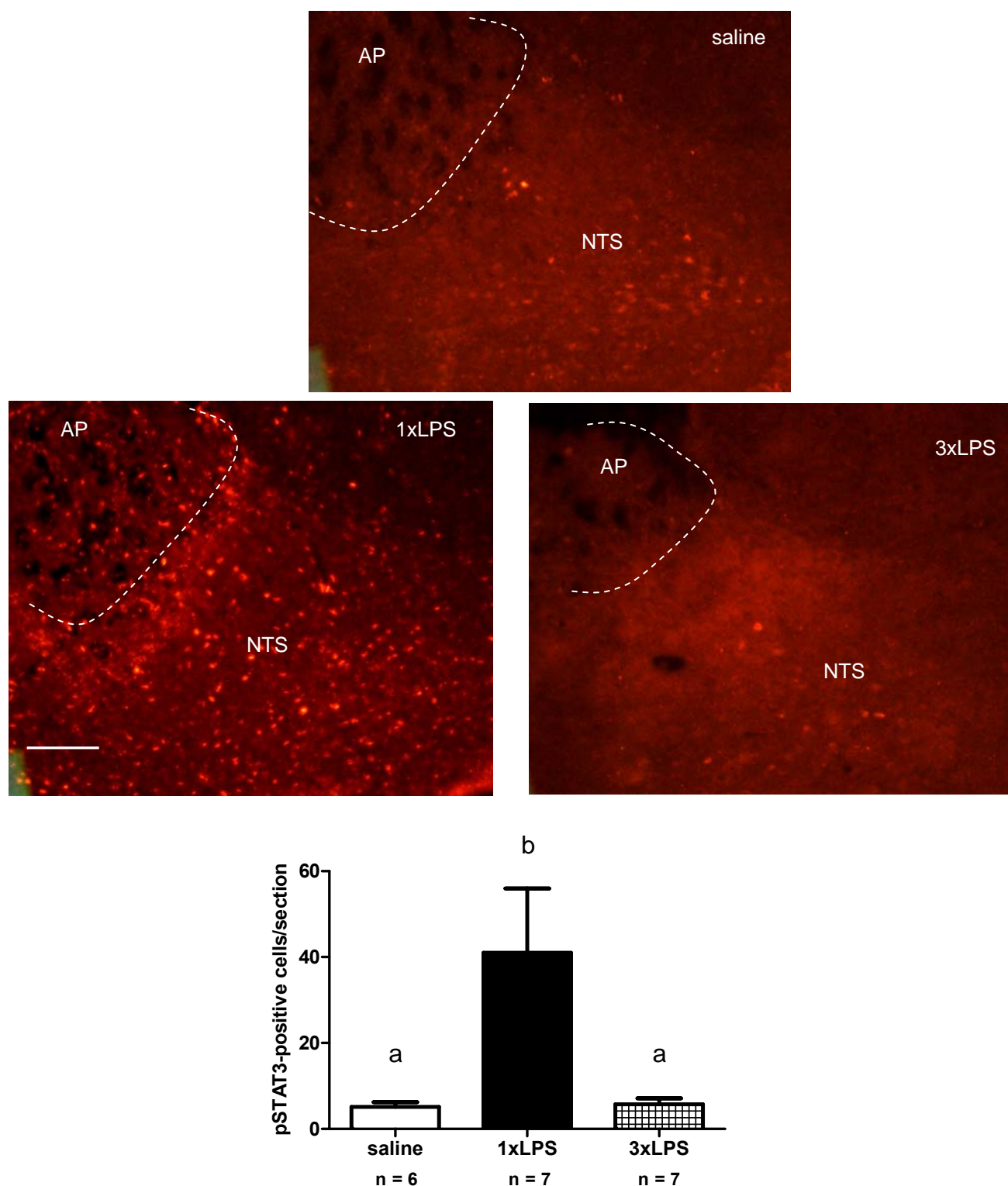


FIG. 15. Effect of repeated injections of LPS (100µg/kg) on pSTAT3 formations in the NTS. The rats received either saline (A; saline/saline/saline), single (B; saline/saline/LPS) or three LPS (100µg/kg IP) injections (C; LPS/LPS/LPS) at dark onset on days 1, 3 and 5. Single LPS treatment induced a significantly higher number of pSTAT3 positive cells than saline; no effect was observed after repeated LPS treatment (D). “AP = area postrema”. “NTS = nucleus tractus solitarii”. Scale bar: 100µm. Different letters mean statistically significant values between groups.

5 Discussion

5.1 Effect of 1400W treatment on LPS-induced anorexia and associated disease symptoms

One aim of the present study was to test the possible involvement of NO in disease-related anorexia. If NO contributed to the LPS-induced reduction of food intake, a pharmacological blockade of iNOS activity by 1400W would be expected to attenuate LPS-induced anorexia. In line with this hypothesis, 1400W injected subcutaneously at a dose of 10mg/kg partly reversed the reduction of food intake in LPS treated animals. Hence, we demonstrated for the first time that iNOS-mediated NO formation appears to be part of the neuromechanisms triggering disease-related anorexia, at least in the LPS inflammation model.

While there is evidence that NO might play a role in the control of food intake under normal, non-pathological conditions, studies investigating the relevance of NO in disease-related anorexia are scarce. In general support of our view that iNOS induction may be pathologically relevant in this context, are observations that brain iNOS expression increases in tumor bearing mice (Wang et al. 2005). Interestingly iNOS expression was shown to be increased in the hypothalamic paraventricular nucleus and the lateral hypothalamic area. These effects seemed to be specifically induced by tumor growth and not by anorexia per se, because healthy pair-fed animals receiving the same amount of food as the anorectic tumor bearing animals did not show this effect. Unfortunately, the ARC was not analyzed in these studies, but a similarly high iNOS expression had been described in the ARC as in the paraventricular nucleus after LPS-induced inflammation (Wong et al. 1996).

In the studies by Wang et al. (Wang et al. 2005), unspecific NOS inhibitors were given via the drinking water to examine a possible effect on cancer anorexia. Cancer anorexia was not affected by this treatment. We believe, however, that it is questionable to exclude a functional role specifically of iNOS signaling in disease-related anorexia based on these experiments. Blockade of NOS with unspecific antagonists is known to decrease food intake after peripheral and central administration in healthy animals (Morley and Flood 1991; Morley and Flood 1992; Squadrito et al. 1993; Morley and Flood 1994; Squadrito et al. 1994). This well-known effect is unlikely to be mediated by iNOS that is hardly expressed under non-pathological conditions in the brain. It is rather thought to be mediated by an inhibition of neuronal NOS (nNOS) but the exact mechanisms are not

fully understood. Irrespective of the underlying mechanisms responsible for the decrease in food intake after unspecific NOS antagonisms at baseline, this effect may compensate or mask an increase in food intake resulting from concomitant iNOS inhibition when a similar approach is used under inflammatory experimental conditions. In contrast to the suppression of feeding by non-selective NOS inhibitors, we observed only a weak and non-significant reduction in food intake after iNOS antagonism at baseline; this in principal suggests the specificity of the iNOS inhibition by 1400W. These results further imply that 1400W treatment does not seem to induce strong side effects that might reduce food intake via aversive mechanisms, such as e.g. hemodynamic effects that occur after unspecific NOS inhibition (Torok 2008).

Similar to food intake, the reduction of water intake after LPS treatment was also attenuated by 1400W. Irrespective of the question whether this effect is directly mediated by the inhibition of NO formation or whether it is secondary to the attenuated anorexia, a reversal of adipsia is a beneficial effect contributing to the amelioration of disease-related symptoms. Adipsia and dehydration are common clinical problems during inflammation (Hart 1988).

The subfornical organ (SFO) belongs to the circumventricular organs and has been implicated in NO-dependent effects on drinking behavior. In 1975, it was described that injection of the artificial NO donor sodium nitroprusside (SNP) into the SFO exerts an antidipsogenic effect on angiotensin II induced drinking (Nicolaidis and Fitzsimons 1975). In line with this observation, SNP inhibits angiotensin II sensitive SFO neurons via cGMP formation (Rauch et al. 1997). An SFO intrinsic NO formation following strong excitatory stimuli (e.g. angiotensin II) has been suggested as an inhibitory feedback mechanism limiting neuronal excitability. Although this proposed neuromechanism primarily involves nNOS, it appears plausible that an iNOS-mediated NO formation in the SFO could also lead to an inhibition of dipsogenic angiotensin II sensitive neurons. Similar to the pSTAT3 response observed in the ARC, LPS induces a STAT3 translocation in the SFO (Rummel et al. 2004). Based on the role of the JAK-STAT signaling in the transcriptional regulation of iNOS expression (see below), these findings suggest that LPS and other inflammatory stimuli might reduce drinking via an iNOS-dependent NO-formation in the SFO.

Our current findings that iNOS antagonism attenuates the LPS-induced adipsia are compatible with such a mechanism; however, the possibility of an indirect mediation, e.g. subsequent to reduced eating after LPS, cannot be excluded.

In addition to the reversal of reduced eating and drinking after LPS, the LPS-induced hyperthermia was partly reversed by 1400W treatment. NO plays a crucial role in the

induction of hyperthermia (Parrott et al. 1998) (Roth et al. 1998). Our data are in line with studies showing that the NOS inhibitor L-NAME significantly attenuated LPS-induced hyperthermia (Soszynski 2001). Furthermore Roth et al. demonstrated a dose-dependent antipyretic effect of the selective iNOS inhibitors aminoguanidine and S-methylisothiourea (Roth et al. 1999). Hence, since the attenuated hyperthermia response after 1400W was expected, this observation might be considered as an internal positive control confirming the effectiveness of 1400W to reverse iNOS mediated actions under our experimental conditions.

Lethargy and reduced physical activity belong to the most commonly recognized behavioral patterns of sick animals (Hart 1988). The LPS-induced reduction in locomotor activity was also partly reversed by 1400W treatment. Hence, the higher activity of LPS/1400W-injected rats might be interpreted as improved general health status and is in line with the increased food intake and attenuated hyperthermia after 1400W. The neuronal mechanisms involved in LPS-induced lethargy and inactivity are poorly understood. Interestingly, in our previous studies LPS reduced c-Fos expression in orexin positive neurons of the lateral hypothalamic area (Becskei et al. 2008). Orexins are implicated in the control of sleep and arousal (Chemelli et al. 1999). While the possible function of NO formation in the LHA for activity has not yet been explored specifically, it is known that nNOS expressing neurons are co-distributed with orexin neurons (Cutler et al. 2001). Furthermore, iNOS expression is upregulated in the LHA of tumor bearing rats (Wang et al. 2005). Hence, it is in principal plausible that a NO-dependent reduction of neuronal activity in orexinergic LHA neurons might be involved in both LPS-induced anorexia and inhibition of locomotor activity.

LPS treatment reduced energy expenditure, and 1400W partly reversed this effect. We assume that the LPS-mediated reduction in energy expenditure is at least in part a consequence of reduced physical activity and food intake. To our knowledge, there are no published studies directly investigating the involvement of NO in energy expenditure. A specific effect on energy expenditure could e.g. be investigated in pair-feeding experiments. It might be more difficult to control for differences in locomotor activity, because pair feeding of healthy controls to LPS treated animals is likely to increase physical activity in control animals that are pair-fed to LPS treated animals because of increased food seeking behavior.

Beside energy expenditure we also determined the effect of LPS on the respiratory quotient (RQ). Possibly due to the strong reduction of food intake, the RQ of LPS-treated animals was between 0,7 and 0,8, whereas the RQ of the control animals was about 0,9. This indicates a shift towards lipid metabolism in LPS treated rats. Despite a partial

reversal of LPS-induced anorexia, 1400W had no obvious influence on the LPS-induced reduction of the RQ. A likely explanation is that the partial reversal of LPS anorexia did not compensate sufficiently for the negative energy balance to shift back from fat towards carbohydrate metabolism.

In summary our data indicate that NO seems to be involved in LPS-induced symptoms such as disease-related anorexia and some other disease-related symptoms (e.g. adipisia, inactivity, fever). An inhibition of iNOS-mediated NO formation by the long acting iNOS inhibitor 1400W might be a therapeutic approach to treat illness anorexia.

5.2 LPS-induced pSTAT3 formation in the ARC

In the second part of this project we characterized the temporal pattern of LPS on pSTAT3 formation in the ARC and AP/NTS region after LPS-treatment. Previous semiquantitative studies had demonstrated that LPS triggers a time-dependent nuclear STAT3 translocation in the ARC and other brain structures of guinea pigs (Rummel et al. 2005) and rats (Gautron et al. 2002; Rummel et al. 2005). However, to our knowledge there are no immunohistochemical studies quantifying the LPS-induced pSTAT3 response in the ARC of rats after different exposure times. Further, we specifically detected the phosphorylated STAT3 molecule, which represents the activated and functional form upon receptor activation. LPS treated rats showed a markedly enhanced number of pSTAT3 positive cells in the ARC and the AP/NTS region 4h, but not 2h after treatment relative to saline-injected controls. Our data are in principle consistent with previous findings demonstrating that peripheral LPS administration induced nuclear translocation of STAT3 in the hypothalamus peaking between 2 and 4 h after injection (Gautron et al. 2002). In addition, the time dependent STAT3 activation in the hypothalamus after LPS-injections has also been investigated using Western blot studies in hypothalamic tissue probes, showing that LPS-induced STAT3 phosphorylation started 2 h and peaked 4 hours after LPS treatment (Hosoi et al. 2004).

pSTAT3 formation in the brain is stimulated by different cytokines which are the putative endogenous mediators of LPS-induced STAT signaling (Imada and Leonard 2000). IL-1 β (Samavati et al. 2009) and IL-6 (Zhong et al. 1994) are potent stimulators of the STAT3 pathway after peripheral (Harre et al. 2002) administration. It is therefore well accepted that these and probably other cytokines may contribute to LPS-induced STAT3 activation in the brain.

We observed pSTAT3 positive cells, i.e. cytoplasmatic pSTAT3 immunoreactivity in the ARC under basal conditions. Leptin is known to induce STAT3 phosphorylation in ARC neurons (Hakansson and Meister 1998), but it remains to be determined whether the basal

pSTAT3 formation in general is driven by leptin. The reason why the total number of pSTAT3 positive cells 2 hours after treatments was slightly but significantly lower in LPS treated rats than in controls also remains unknown. An excessive NO formation has been shown to attenuate the leptin-mediated STAT3 activation (Jang et al. 2007), but this mechanism is unlikely to account for the reduced pSTAT3 signaling, assuming that the LPS-induced iNOS is not yet strongly expressed at this timepoint (Wong et al. 1996).

It was striking that the shape of the pSTAT3-positive cellular signal was different from basal pSTAT3 immunoreactivity. The staining pattern of activated cells 4 hours after the LPS treatment looked smaller and more elongate, while the staining pattern of activated cells after 2 hours LPS or saline treatment appeared bigger and more roundish. Similar findings have been described in studies investigating total (non-phosphorylated and phosphorylated) STAT3 immunoreactivity in the ARC of LPS treated rats. This change in the cellular signal is thought to reflect the translocation of pSTAT3 after its dimerization into the nucleus because STAT3 immunoreactivity colocalizes with DAPI nuclear staining after different inflammatory stimuli (Gautron et al. 2005).

We did not yet identify the phenotype of LPS responsive cells. There is evidence that the STAT3 response is induced by LPS in endothelial cells which are positive for von Willebrand factor and in astrocytes which are positive for glial fibrillary acidic protein (GFAP) (Rummel et al. 2005) in the ARC of guinea pigs. Whether these cell types are the only target cells and which proportion of these subsets of cells shows a LPS-induced pSTAT response has not yet been determined in detail. In our own preliminary double-labeling studies, we did not detect a colocalization of pSTAT3 and GFAP in the ARC of LPS treated rats, indicating that at least under our conditions, astrocytes do not seem to be the major cell type responding to LPS with a pSTAT3 formation in the ARC of rats.

In addition to endothelial cells and possibly astrocytes, microglia cells might also respond with a pSTAT3 response after inflammatory stimuli. Microglia cells are the resident immune cells of the brain and constantly patrol the cerebral microenvironment to respond to pathogens and damage (Perry et al. 1995). A single systemic injection of LPS results in a robust expression of pro-inflammatory cytokines and chemokines in microglia cells (Beutler 2000). Cytokines upregulate iNOS expression in microglia and thus produce nitric oxide after LPS exposure (Possel et al. 2000). It has also been reported that injection of LPS induces a concentration and time-dependent expression of iNOS in activated microglia in the brain of adult rats, with an induction of iNOS protein 4 hours after LPS injection (Arimoto and Bing 2003). A colocalization of pSTAT3 and the microglia cell marker CD11b has been established in thrombin-induced microglia activation (Huang et al. 2008) suggesting a functional link between STAT3 signaling and

iNOS expression in microglia cells. Whether an LPS-induced STAT3 phosphorylation occurs in microglia cells of the ARC and the AP/NTS region under our conditions awaits further investigation.

5.3 Association between pSTAT formation and LPS-induced anorexia

Based on the previous experiment, a follow-up study with repeated LPS injections was performed to investigate a possible association between pSTAT3 formation and LPS-induced anorexia. We analyzed pSTAT3 formation in the ARC and AP/NTS-region after a single or after repeated injection of LPS. We demonstrated a higher number of pSTAT3 positive cells in these brain areas after a single LPS treatment compared to controls, but the number of pSTAT3 positive cells was similar to control conditions after repeated LPS injection. Similar to the blunted pSTAT3 formation in the brain, there was no hypophagic effect of LPS after repeated intraperitoneal injections; LPS did no longer reduce food intake compared to control animals after two prior LPS injections given over three days. This was expected based on previous studies under similar experimental condition (Langhans et al. 1991).

Our study is the first to indicate that the LPS-induced pSTAT3 formation in the ARC and the AP/NTS region may be a cellular correlate of the anorectic effect to LPS. Our observations are consistent with the idea that pSTAT3 signaling may be part of the intracellular signaling mechanisms mediating LPS anorexia, because repeated LPS injections appeared to produce tolerance towards both effects. It has to be noted, however, that the blunted pSTAT3 response after repeated LPS treatment is most likely a generalized effect also occurring in other brain areas and affecting other disease-related symptoms. Hence, to confirm that pSTAT3 formation is functionally involved in LPS anorexia will require further studies.

While there is evidence that the ARC is involved in anorexia caused by inflammatory stimuli (Reyes and Sawchenko 2002) the importance of STAT signaling in this context has not yet been assessed. Intracerebroventricular injection of the JAK inhibitor AG-490 has been shown to block the anorectic action of icv administered leptin (Morrison et al. 2007), which acts via ARC neurons to reduce feeding. Hence, this compound could also be useful to prove the functional involvement of the JAK-STAT pathway in LPS anorexia.

The role of LPS-induced pSTAT3 signaling in AP/NTS is not well understood and the same is true for the general importance of this brain area in disease-related anorexia. The AP/NTS region is a site of convergence of humoral signals acting on AP neurons and of

signals transmitted via the vagus nerve. Depending on the dose and route of administration, the LPS and IL-1 β induced anorexia was attenuated by subdiaphragmatic vagotomy (Bluthe et al. 1996) (Schwartz et al. 1997), while total ablation of the AP/NTS region appeared to enhance LPS-induced anorexia (Weingarten et al. 1993). Similar to other circumventricular organs, the AP is considered a neural-immune interface showing a variety of cellular responses after immune challenges including the expression of IL-1 β (Goehler et al. 2006). The AP/NTS region might also be involved in stress responses and cardiovascular effects induced by LPS and cytokines. An AP lesion has been shown to abolish the stimulatory effect of IL-1 β on ACTH and corticosterone plasma levels (Lee et al. 1998). Interestingly, LPS induces an iNOS dependent NO release in the NTS, which seems to mediate LPS-induced, delayed hypotension (Lin et al. 1999). Based on the established role of the JAK-STAT pathway in the transcriptional regulation of iNOS expression, it is conceivable that the LPS-induced STAT phosphorylation is involved in these processes. As proposed above, a specific inhibition of the JAK-STAT pathway, ideally via a site specific injection of JAK inhibitors, might be a promising approach to confirm the relevance of STAT signaling not only for the expression of iNOS but also for the manifestation of pathological, NO-mediated symptoms.

5.4 Interaction between the pSTAT/iNOS pathway and COX-2 signaling

It is well accepted that inflammatory neuronal mechanisms causing anorexia at least partly depend on the COX-2 pathway, since pharmacological or genetic disruption of COX-2 signaling attenuates the anorectic response in LPS-treated animals (Johnson et al. 2002; Lugarini et al. 2002). COX-2 is upregulated by the transcription factor NF- κ B (Appleby et al. 1994), e.g. in response to IL-1 β (Nadjar et al. 2003). The STAT3 pathway seems to be linked to the activation of COX-2 expression in sensory circumventricular organs including the AP (Rummel et al. 2006) because LPS-induced pSTAT3-immunoreactivity colocalized with COX-2 expression. Interestingly, pSTAT1/3 signaling is involved in the regulation of COX-2 expression because the JAK/STAT inhibitor AG-490 blocked myocardial COX-2 expression (Xuan et al. 2003). The same inhibitor also blunted the LPS/INF γ induced iNOS expression in bovine chromaffine cells (Perez-Rodriguez et al. 2009).

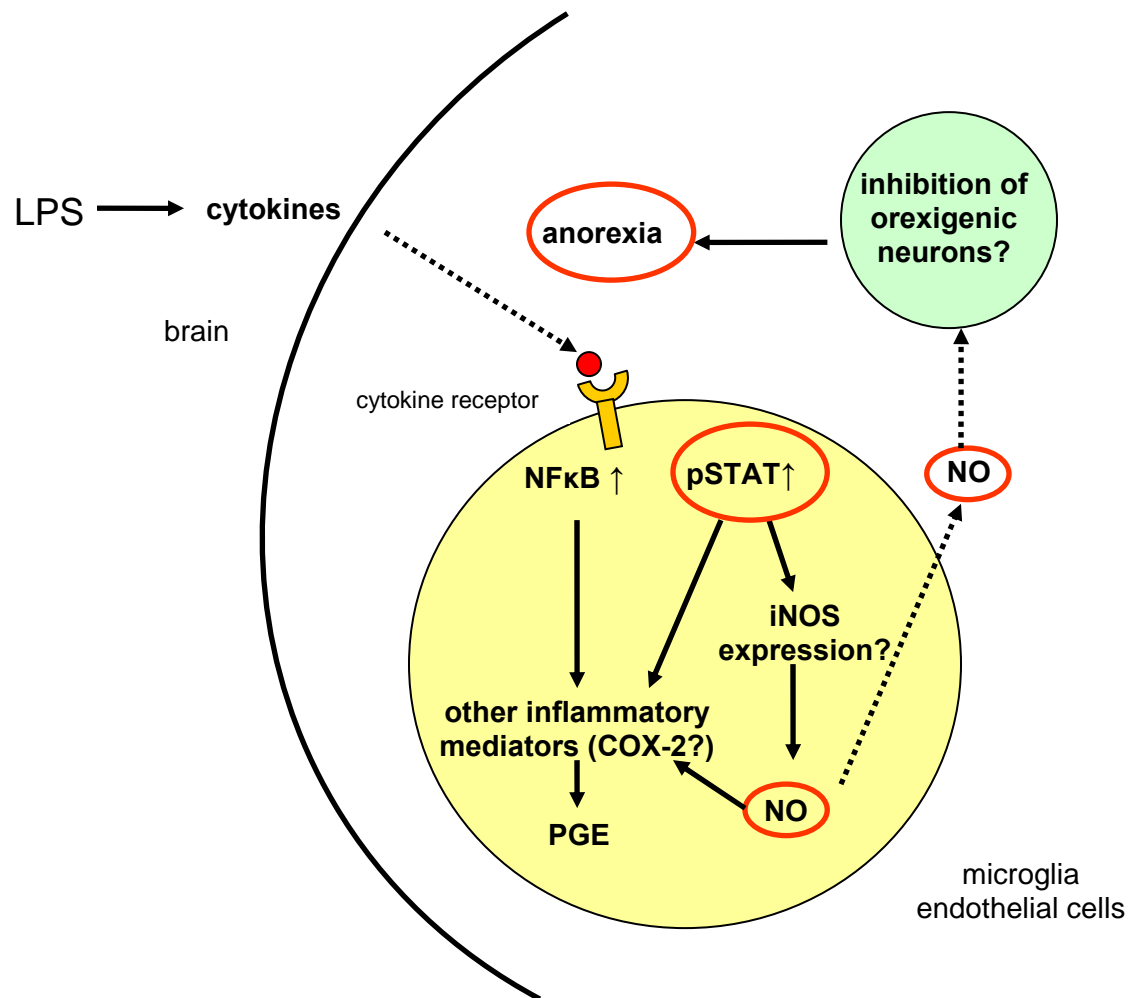
In addition to the possible involvement of pSTAT3 in iNOS expression it is well established that pSTAT1 is an important regulator of iNOS gene transcription. The iNOS promoter region is flanked by cytokine responsive DNA motifs. One of these binds STAT1 and upregulates iNOS gene expression (Guo et al. 2007). A STAT1-mediated

induction of iNOS expression by cytokines and LPS has been demonstrated in different cell systems including astroglial and epithelial cells (Dell'Albani et al. 2001; Stempelj et al. 2007). Hence, based on the aforementioned considerations the transcriptional targets of the JAK-STAT signaling may comprise both, the anorectic COX-2 pathway and an upregulation of iNOS, which contributes to LPS anorexia according to our present studies.

In addition to a common transcriptional regulation, COX-2 and iNOS seem to directly interact at the functional level. COX-2 and iNOS are often expressed together in inflamed tissue and nitric oxide produced by iNOS increases the enzymatic activity of COX-2 (Salvemini et al. 1996). Therefore, it appears plausible that a pSTAT/iNOS dependent NO formation in the ARC might contribute to a reduction in food intake partly directly and partly indirectly via an enhancement of COX-2 activity.

5.5 Proposed model

In summary we propose the following model, which combines our present findings that NO appears to partly mediate LPS-induced anorexia with the established role of COX-2 in this respect. We assume that cytokines trigger a STAT phosphorylation which leads to the induction of iNOS expression and ultimately to NO formation. NO then diffuses to neighboring cells to inhibit orexigenic neurons. As an indirect mechanism NO might also enhance the activity of COX-2 causing an enhancement of PGE2 synthesis. Activation of the JAK-STAT pathway might also contribute to the expression of COX-2 in parallel to the induction of iNOS. Due to the central role of NO as an important pro-inflammatory mediator iNOS might be therapeutically targeted by iNOS inhibitors to treat disease-related anorexia and associated symptoms.



6 References

- Appleby, S. B., A. Ristimäki, et al. (1994). "Structure of the human cyclo-oxygenase-2 gene." Biochem J **302** (Pt 3): 723-7.
- Arimoto, T. and G. Bing (2003). "Up-regulation of inducible nitric oxide synthase in the substantia nigra by lipopolysaccharide causes microglial activation and neurodegeneration." Neurobiol Dis **12**(1): 35-45.
- Barsh, G. S. and M. W. Schwartz (2002). "Genetic approaches to studying energy balance: perception and integration." Nat Rev Genet **3**(8): 589-600.
- Becskei, C., T. Riediger, et al. (2008). "Inhibitory effects of lipopolysaccharide on hypothalamic nuclei implicated in the control of food intake." Brain Behav Immun **22**(1): 56-64.
- Beutler, B. (2000). "Tlr4: central component of the sole mammalian LPS sensor." Curr Opin Immunol **12**(1): 20-6.
- Bluthe, R. M., B. Michaud, et al. (1996). "Vagotomy attenuates behavioural effects of interleukin-1 injected peripherally but not centrally." Neuroreport **7**(9): 1485-8.
- Chai, Z., S. Gatti, et al. (1996). "Interleukin (IL)-6 gene expression in the central nervous system is necessary for fever response to lipopolysaccharide or IL-1 beta: a study on IL-6-deficient mice." J Exp Med **183**(1): 311-6.
- Chemelli, R. M., J. T. Willie, et al. (1999). "Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation." Cell **98**(4): 437-51.
- Cowley, M. A., J. L. Smart, et al. (2001). "Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus." Nature **411**(6836): 480-4.
- Cutler, D. J., R. Morris, et al. (2001). "Orexin-A immunoreactive neurons in the rat hypothalamus do not contain neuronal nitric oxide synthase (nNOS)." Peptides **22**(1): 123-8.
- Czapski, G. A., M. Cakala, et al. (2007). "Role of nitric oxide in the brain during lipopolysaccharide-evoked systemic inflammation." J Neurosci Res **85**(8): 1694-703.
- Dantzer, R. (2006). "Cytokine, sickness behavior, and depression." Neurol Clin **24**(3): 441-60.
- Dell'Albani, P., R. Santangelo, et al. (2001). "JAK/STAT signaling pathway mediates cytokine-induced iNOS expression in primary astroglial cell cultures." J Neurosci Res **65**(5): 417-24.
- Fattori, E., M. Cappelletti, et al. (1994). "Defective inflammatory response in interleukin 6-deficient mice." J Exp Med **180**(4): 1243-50.
- Ferguson, A. V. (1991). "The area postrema: a cardiovascular control centre at the blood-brain interface?" Can J Physiol Pharmacol **69**(7): 1026-34.
- Garvey, E. P., J. A. Oplinger, et al. (1997). "1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo." J Biol Chem **272**(8): 4959-63.
- Gautron, L., P. Lafon, et al. (2002). "Spatiotemporal analysis of signal transducer and activator of transcription 3 activation in rat brain astrocytes and pituitary following peripheral immune challenge." Neuroscience **112**(3): 717-29.
- Gautron, L., R. Mingam, et al. (2005). "Influence of feeding status on neuronal activity in

- the hypothalamus during lipopolysaccharide-induced anorexia in rats." Neuroscience **134**(3): 933-46.
- Goehler, L. E., A. Erisir, et al. (2006). "Neural-immune interface in the rat area postrema." Neuroscience **140**(4): 1415-34.
- Guo, Z., L. Shao, et al. (2007). "Identification of a classic cytokine-induced enhancer upstream in the human iNOS promoter." Faseb J **21**(2): 535-42.
- Hakansson, M. L. and B. Meister (1998). "Transcription factor STAT3 in leptin target neurons of the rat hypothalamus." Neuroendocrinology **68**(6): 420-7.
- Harre, E. M., J. Roth, et al. (2002). "Selected contribution: role of IL-6 in LPS-induced nuclear STAT3 translocation in sensory circumventricular organs during fever in rats." J Appl Physiol **92**(6): 2657-66.
- Hart, B. L. (1988). "Biological basis of the behavior of sick animals." Neurosci Biobehav Rev **12**(2): 123-37.
- Hebenstreit, D., J. Horejs-Hoeck, et al. (2005). "JAK/STAT-dependent gene regulation by cytokines." Drug News Perspect **18**(4): 243-9.
- Horvath, T. L., S. Diano, et al. (2001). "Minireview: ghrelin and the regulation of energy balance--a hypothalamic perspective." Endocrinology **142**(10): 4163-9.
- Hosoi, T., Y. Okuma, et al. (2004). "Bacterial endotoxin induces STAT3 activation in the mouse brain." Brain Res **1023**(1): 48-53.
- Huang, C., R. Ma, et al. (2008). "JAK2-STAT3 signaling pathway mediates thrombin-induced proinflammatory actions of microglia in vitro." J Neuroimmunol **204**(1-2): 118-25.
- Ignarro, L. J. (1991). "Signal transduction mechanisms involving nitric oxide." Biochem Pharmacol **41**(4): 485-90.
- Imada, K. and W. J. Leonard (2000). "The Jak-STAT pathway." Mol Immunol **37**(1-2): 1-11.
- Jafarian-Tehrani, M., G. Louin, et al. (2005). "1400W, a potent selective inducible NOS inhibitor, improves histopathological outcome following traumatic brain injury in rats." Nitric Oxide **12**(2): 61-9.
- Jang, E. H., C. S. Park, et al. (2007). "Excessive nitric oxide attenuates leptin-mediated signal transducer and activator of transcription 3 activation." Life Sci **80**(7): 609-17.
- Johnson, P. M., S. K. Vogt, et al. (2002). "COX-2 inhibition attenuates anorexia during systemic inflammation without impairing cytokine production." Am J Physiol Endocrinol Metab **282**(3): E650-6.
- Kim, O. S., E. J. Park, et al. (2002). "JAK-STAT signaling mediates gangliosides-induced inflammatory responses in brain microglial cells." J Biol Chem **277**(43): 40594-601.
- Kim, Y. W., K. H. Kim, et al. (2007). "Time-course changes of hormones and cytokines by lipopolysaccharide and its relation with anorexia." J Physiol Sci **57**(3): 159-65.
- Kinoshita, D., D. W. Cohn, et al. (2009). "Behavioral effects of LPS in adult, middle-aged and aged mice." Physiol Behav **96**(2): 328-32.
- Kojima, M., H. Hosoda, et al. (1999). "Ghrelin is a growth-hormone-releasing acylated peptide from stomach." Nature **402**(6762): 656-60.
- Korhonen, R., A. Lahti, et al. (2005). "Nitric oxide production and signaling in inflammation." Curr Drug Targets Inflamm Allergy **4**(4): 471-9.

- Kozak, W. and A. Kozak (2003). "Genetic Models in Applied Physiology. Differential role of nitric oxide synthase isoforms in fever of different etiologies: studies using Nos gene-deficient mice." J Appl Physiol **94**(6): 2534-44.
- Langhans, W. (2007). "Signals generating anorexia during acute illness." Proc Nutr Soc **66**(3): 321-30.
- Langhans, W., G. Balkowski, et al. (1991). "Differential feeding responses to bacterial lipopolysaccharide and muramyl dipeptide." Am J Physiol **261**(3 Pt 2): R659-64.
- Langhans, W. and B. Hrupka (1999). "Interleukins and tumor necrosis factor as inhibitors of food intake." Neuropeptides **33**(5): 415-24.
- Langhans, W., D. Savoldelli, et al. (1993). "Comparison of the feeding responses to bacterial lipopolysaccharide and interleukin-1 beta." Physiol Behav **53**(4): 643-9.
- Lee, H. Y., M. B. Whiteside, et al. (1998). "Area postrema removal abolishes stimulatory effects of intravenous interleukin-1beta on hypothalamic-pituitary-adrenal axis activity and c-fos mRNA in the hypothalamic paraventricular nucleus." Brain Res Bull **46**(6): 495-503.
- Lim, C. P. and X. Cao (2006). "Structure, function, and regulation of STAT proteins." Mol Biosyst **2**(11): 536-50.
- Lin, H. C., F. J. Wan, et al. (1999). "Systemic administration of lipopolysaccharide induces release of nitric oxide and glutamate and c-fos expression in the nucleus tractus solitarius of rats." Hypertension **33**(5): 1218-24.
- Lugarini, F., B. J. Hrupka, et al. (2002). "A role for cyclooxygenase-2 in lipopolysaccharide-induced anorexia in rats." Am J Physiol Regul Integr Comp Physiol **283**(4): R862-8.
- Lutz, T. A. (2006). "Amylinergic control of food intake." Physiol Behav **89**(4): 465-71.
- Lutz, T. A., M. Senn, et al. (1998). "Lesion of the area postrema/nucleus of the solitary tract (AP/NTS) attenuates the anorectic effects of amylin and calcitonin gene-related peptide (CGRP) in rats." Peptides **19**(2): 309-17.
- Minghetti, L., A. Nicolini, et al. (1997). "Inducible nitric oxide synthase expression in activated rat microglial cultures is downregulated by exogenous prostaglandin E2 and by cyclooxygenase inhibitors." Glia **19**(2): 152-60.
- Moncada, S., R. M. Palmer, et al. (1991). "Nitric oxide: physiology, pathophysiology, and pharmacology." Pharmacol Rev **43**(2): 109-42.
- Morley, J. E. and J. F. Flood (1991). "Evidence that nitric oxide modulates food intake in mice." Life Sci **49**(10): 707-11.
- Morley, J. E. and J. F. Flood (1992). "Competitive antagonism of nitric oxide synthetase causes weight loss in mice." Life Sci **51**(16): 1285-9.
- Morley, J. E. and J. F. Flood (1994). "Effect of competitive antagonism of NO synthetase on weight and food intake in obese and diabetic mice." Am J Physiol **266**(1 Pt 2): R164-8.
- Morrison, C. D., C. L. White, et al. (2007). "Increased hypothalamic protein tyrosine phosphatase 1B contributes to leptin resistance with age." Endocrinology **148**(1): 433-40.
- Nadjar, A., C. Combe, et al. (2003). "Nuclear factor kappaB nuclear translocation as a crucial marker of brain response to interleukin-1. A study in rat and interleukin-1 type I deficient mouse." J Neurochem **87**(4): 1024-36.
- Nicolaidis, S. and J. Fitzsimons (1975). La dependance de la prise d'eau induit par

- l'angiotensin II envers la fonction vasomotrice cerebrale locale chez le rat. CR Acad Soc Paris **281**: 1417-1420.
- Parmentier, S., G. A. Bohme, et al. (1999). "Selective inhibition of inducible nitric oxide synthase prevents ischaemic brain injury." Br J Pharmacol **127**(2): 546-52.
- Parrott, R. F., S. V. Vellucci, et al. (1998). "Effects of intravenous nitric oxide inhibitors on endotoxin-induced fever in prepubertal pigs." Gen Pharmacol **31**(3): 371-6.
- Perez-Rodriguez, R., C. Roncero, et al. (2009). "Signaling mechanisms of interferon gamma induced apoptosis in chromaffin cells: involvement of nNOS, iNOS, and NFkappaB." J Neurochem **108**(4): 1083-96.
- Perry, V. H., M. D. Bell, et al. (1995). "Inflammation in the nervous system." Curr Opin Neurobiol **5**(5): 636-41.
- Plata-Salaman, C. R. (1996). "Anorexia during acute and chronic disease." Nutrition **12**(2): 69-78.
- Plata-Salaman, C. R. (2000). "Central nervous system mechanisms contributing to the cachexia-anorexia syndrome." Nutrition **16**(10): 1009-12.
- Porter, M. H., B. J. Hrupka, et al. (2000). "Inhibition of TNF-alpha production contributes to the attenuation of LPS-induced hypophagia by pentoxifylline." Am J Physiol Regul Integr Comp Physiol **279**(6): R2113-20.
- Possel, H., H. Noack, et al. (2000). "Selective upregulation of inducible nitric oxide synthase (iNOS) by lipopolysaccharide (LPS) and cytokines in microglia: in vitro and in vivo studies." Glia **32**(1): 51-9.
- Rauch, M., H. A. Schmid, et al. (1997). "Electrophysiological and immunocytochemical evidence for a cGMP-mediated inhibition of subfornical organ neurons by nitric oxide." J Neurosci **17**(1): 363-71.
- Reyes, T. M. and P. E. Sawchenko (2002). "Involvement of the arcuate nucleus of the hypothalamus in interleukin-1-induced anorexia." J Neurosci **22**(12): 5091-9.
- Ricardo, J. A. and E. T. Koh (1978). "Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat." Brain Res **153**(1): 1-26.
- Riediger, T., P. Giannini, et al. (2006). "Nitric oxide directly inhibits ghrelin-activated neurons of the arcuate nucleus." Brain Res **1125**(1): 37-45.
- Riediger, T., H. A. Schmid, et al. (2001). "Amylin potently activates AP neurons possibly via formation of the excitatory second messenger cGMP." Am J Physiol Regul Integr Comp Physiol **281**(6): R1833-43.
- Roth, J., B. Storr, et al. (1999). "Dose-dependent attenuation of lipopolysaccharide-fever by inhibitors of inducible nitric oxide-synthase in guinea pigs." Eur J Pharmacol **383**(2): 177-87.
- Roth, J., B. Storr, et al. (1998). "Inhibition of nitric oxide synthase attenuates lipopolysaccharide-induced fever without reduction of circulating cytokines in guinea-pigs." Pflugers Arch **436**(6): 858-62.
- Rummel, C., T. Hubschle, et al. (2004). "Nuclear translocation of the transcription factor STAT3 in the guinea pig brain during systemic or localized inflammation." J Physiol **557**(Pt 2): 671-87.
- Rummel, C., C. Sachot, et al. (2006). "Circulating interleukin-6 induces fever through a STAT3-linked activation of COX-2 in the brain." Am J Physiol Regul Integr Comp Physiol **291**(5): R1316-26.

- Rummel, C., T. Voss, et al. (2005). "Nuclear STAT3 translocation in guinea pig and rat brain endothelium during systemic challenge with lipopolysaccharide and interleukin-6." J Comp Neurol **491**(1): 1-14.
- Salvemini, D., Z. Q. Wang, et al. (1996). "Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation." Br J Pharmacol **118**(4): 829-38.
- Samavati, L., R. Rastogi, et al. (2009). "STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria." Mol Immunol **46**(8-9): 1867-77.
- Schwartz, G. J., C. R. Plata-Salaman, et al. (1997). "Subdiaphragmatic vagal deafferentation fails to block feeding-suppressive effects of LPS and IL-1 beta in rats." Am J Physiol **273**(3 Pt 2): R1193-8.
- Soszynski, D. (2001). "The inhibition of nitric oxide synthase suppresses LPS- and psychological-stress-induced fever in rats." Physiol Behav **72**(1-2): 65-72.
- Spanswick, D., M. A. Smith, et al. (1997). "Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels." Nature **390**(6659): 521-5.
- Sparrow, J. R. (1994). "Inducible nitric oxide synthase in the central nervous system." J Mol Neurosci **5**(4): 219-29.
- Squadrito, F., G. Calapai, et al. (1994). "Central serotonergic system involvement in the anorexia induced by NG-nitro-L-arginine, an inhibitor of nitric oxide synthase." Eur J Pharmacol **255**(1-3): 51-5.
- Squadrito, F., G. Calapai, et al. (1993). "Anorectic activity of NG-nitro-L-arginine, an inhibitor of brain nitric oxide synthase, in obese Zucker rats." Eur J Pharmacol **230**(1): 125-8.
- Stamler, J. S., D. J. Singel, et al. (1992). "Biochemistry of nitric oxide and its redox-activated forms." Science **258**(5090): 1898-902.
- Stempelj, M., M. Keding, et al. (2007). "Essential role of the JAK/STAT1 signaling pathway in the expression of inducible nitric-oxide synthase in intestinal epithelial cells and its regulation by butyrate." J Biol Chem **282**(13): 9797-804.
- Stitt, J. T. and S. G. Shimada (1989). "Enhancement of the febrile responses of rats to endogenous pyrogen occurs within the OVLT region." J Appl Physiol **67**(5): 1740-6.
- Tisdale, M. J. (2009). "Mechanisms of cancer cachexia." Physiol Rev **89**(2): 381-410.
- Torok, J. (2008). "Participation of nitric oxide in different models of experimental hypertension." Physiol Res **57**(6): 813-25.
- Traebert, M., T. Riediger, et al. (2002). "Ghrelin acts on leptin-responsive neurones in the rat arcuate nucleus." J Neuroendocrinol **14**(7): 580-6.
- Tschop, M., D. L. Smiley, et al. (2000). "Ghrelin induces adiposity in rodents." Nature **407**(6806): 908-13.
- Valassi, E., M. Scacchi, et al. (2008). "Neuroendocrine control of food intake." Nutr Metab Cardiovasc Dis **18**(2): 158-68.
- Wang, W., E. Svanberg, et al. (2005). "NOS isoenzyme content in brain nuclei as related to food intake in experimental cancer cachexia." Brain Res Mol Brain Res **134**(2): 205-14.
- Wei, X. Q., I. G. Charles, et al. (1995). "Altered immune responses in mice lacking inducible nitric oxide synthase." Nature **375**(6530): 408-11.

- Weingarten, S., M. Senn, et al. (1993). "Does a learned taste aversion contribute to the anorectic effect of bacterial lipopolysaccharide?" Physiol Behav **54**(5): 961-6.
- West, M. A. and W. Heagy (2002). "Endotoxin tolerance: A review." Crit Care Med **30**(1 Supp): S64-S73.
- Wong, M. L., V. Rettori, et al. (1996). "Inducible nitric oxide synthase gene expression in the brain during systemic inflammation." Nat Med **2**(5): 581-4.
- Wren, A. M., C. J. Small, et al. (2001). "Ghrelin causes hyperphagia and obesity in rats." Diabetes **50**(11): 2540-7.
- Xuan, Y. T., Y. Guo, et al. (2003). "Mechanism of cyclooxygenase-2 upregulation in late preconditioning." J Mol Cell Cardiol **35**(5): 525-37.
- Zeisberger, E. and J. Roth (1998). "Tolerance to pyrogens." Ann N Y Acad Sci **856**: 116-31.
- Zhong, Z., Z. Wen, et al. (1994). "Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6." Science **264**(5155): 95-8.
- Zhu, Y., D. Nikolic, et al. (2004). "Mechanism of Inactivation of Inducible Nitric Oxide Synthase by Amidines. Irreversible Enzyme Inactivation without Inactivator Modification." Journal American Chemical Society **127**: 858-868.

ACKNOWLEDGEMENT

I wish to thank everybody who has contributed to the accomplishment of this dissertation.

I would specially like to thank PD Dr. Thomas Riediger for his support and patience while supervising me on my dissertation and for his competent help for technical and computer-related problems.

Further, I would like to thank Prof. Dr. Thomas A. Lutz for always being friendly and helpful and for giving me the opportunity of writing my dissertation in his group. Moreover, I am also very thankful for the critical examination of this work.

Many thanks go also to PD Dr. Herbert Schmid for the critical reading of this work.

A special thank goes also to Sarah Habegger, Carmen Brazerol and Lettebrhan Ghebre for the care of my animals.

Many thanks goes to Catarina, Christian, Christina, Karoline, Kerstin, Melanie, Melania, Nadine, Andreas and specially Manuela for their help, for teaching me various laboratory techniques and handling of the metabolic cages and above all the great time we had in our office.

Last but not least, I would like to thank my family and my friends for always being there.

Curriculum Vitae

Caroline Arabelle Cordani

10. Mai 1982	born in Zurich Father: Kurt Cordani Mother: Léonie Cordani, born Wallimann Native place: Wangen (SZ), Switzerland
1989-1995	Primary school in Wädenswil (ZH)
1995-1997	Junior high school in Au (ZH)
1997-2002	High school in Zurich, Matura D
2002-2007	Study in veterinary medicine at the Vetsuisse Faculty University of Zurich
Nov. 2007	Graduation in veterinary medicine at the Vetsuisse Faculty University of Zurich
2008-2009	Dissertation at the Institute of Veterinary Physiology of the Vetsuisse Faculty University of Zurich

19.01.2010